Docket No.: 05408/100J111-US2
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Michael Ludensky et al.

Application No.: 10/540,293 Confirmation No.: 8281

Filed: July 20, 2005 Art Unit: 1724

For: METHOD FOR REMOVAL OF BIOFILM Examiner: P. A. Hruskoci

DECLARATION OF MICHAEL LUDENSKY, PH.D.
UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Michael Ludensky, hereby declare and state as follows:

1. I am a citizen of the United States and over the age of 21.

2. I received a M.S. in Biophysics from Dnepropetrovsky University, Dnepropetrovsk, Ukraine in 1974 and a Ph.D. in Environmental Biology from Dnepropetrovsky University in 1984.

3. For almost 15 years while professionally associated with Lonza Inc., I have researched biofilm growth, monitoring, control, and removal in water treatment applications including supporting the commercialization of halohydantoins and quaternary ammonium compounds as biocides. My curriculum vitae is provided as Exhibit A to my Declaration.

4. I am a named inventor and have assigned the invention of the above-captioned application to Lonza Inc.

5. I have read and am familiar with the above-captioned application and its pending claims, as amended in the Amendment submitted herewith. The pending claims recite a method for disintegrating biofilm by adding to or forming in a medium a monochlorodialkylhydantoin, dichlorodialkylhydantoin or a mixture thereof, wherein the chlorinated hydantoin can disintegrate biofouling at a concentration in which BCDMH is unable to disintegrate the biofilm.

6. I understand that the above-captioned application is a national stage application of International Application No. PCT/US2003/017882 filed June 6, 2003. I also understand that the relevant date for evaluating obviousness of the application's pending claims is, at the very latest, this filing date.

7. I further understand that the Examiner has issued an Office Action for the above-captioned application citing U.S. Patent No. 5,662,940 (referenced herein as "Hight") as teaching or suggesting both the control and disintegration of biofilms. I have read and am familiar with Hight.

8. In my opinion, while Hight discloses the control of biofilms, Hight does not teach or suggest the disintegration of biofilms as set forth in the pending claims because one of ordinary skill in the art as of June 6, 2003 would consider the control and disintegration of biofilms as distinct. There are many means for preventing biofilm growth that are totally ineffectual at removing biofilm. Filter sterilization, for example, will prevent biofilm formation but is of no effect at removing it once formed.

9. In a review chapter by Schulte *et al.* ("Efficacy of biocides against biofilms," Chapter 5.1 in *Directory of Microbiocides for the Protection of Materials. A Handbook.* Ed. W. Paulus. Springer, pp.93 – 120, 2005; Exhibit B), the authors assert that "it is most important to distinguish between killing [*i.e.*, control] and cleaning [*i.e.*, removal or disintegration] with cleaning being at least equally important as killing" (*see* page 96, first complete paragraph). These authors explain that, after using a biocide that does not have a cleaning effect, the dead biomass stays in place and provides nutrients for cells that enter the system later. In such cases, disinfection does not solve the problems but instead leads to rapid regrowth and additional biofilm development. The authors conclude that such effect "is frequently observed in practice and represents the cause of failures of

anti-fouling measures using disinfectants and biocides" (*see* page 96, first complete paragraph). Authors separately address biocidal versus cleaning effects in their chapter. *See, e.g.*, subchapter 5.1.4.3.4. and subchapter 5.1.5.1 (describing experiments using chlorine for biofilm control and cleaning and demonstrating that many chlorine species are not effective at biofilm cleaning at concentrations that are relevant in practice).

10. I have developed several biofilm monitoring techniques to differentiate between biofilm removal and biofilm control. I described one such technique in which heat transfer resistance (HTR) is used to measure biofilm accumulation (dead or alive) and the dissolved oxygen concentration reflects biocidal efficacy (*Journal of Industrial Microbiology and Biotechnology* 1998, 20:109-115, Exhibit C). Using this technique, I demonstrated that most oxidizing and non-oxidizing biocides do not initiate biofilm removal which could have been observed by a significant reduction of HTR, but some biofilm control is observed as measured by dissolved oxygen concentrations (Ludensky, M.L., *International Biodeterioration and Biodegradation* 2003, 51:255-263, Exhibit D). Results using exclusively chlorinated hydantoins were not presented in this article.

11. Thus, the control and disintegration of biofilms are distinct as demonstrated by several scientific publications including articles showing that different techniques, HTR and dissolved oxygen, are used to measure disintegration and control, respectively. These articles further show that not all biocides disintegrate biofilms. In my opinion, Hight merely discloses the control of biofilms using biocides, but does not teach or suggest the disintegration of biofilms using the same.

12. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the instant application or any patent issued thereupon.

Respectfully submitted,

Dated: 10/1/2007

M. Ludensky

Michael Ludensky, Ph.D.

Exhibits:

A. Curriculum Vitae of Dr. Michael L. Ludensky

B. Schulte, S., J. Wingender and H-C. Flemming. (2005) "Efficacy of biocides against biofilms" Chapter 5.1 in *Directory of Microbiocides for the Protection of Materials. A Handbook.* Ed.W. Paulus. Springer, pp.93 – 120.

C. Ludensky, M.L., *Journal of Industrial Microbiology and Biotechnology* 1998, 20:109-115.

D. Ludensky, M.L., 2003, *International Biodeterioration and Biodegradation* 2003, 51:255-263.

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DECLARATION OF MICHAEL LUDENSKY, PH.D. UNDER 37 C.F.R. § 1.132

Exhibit A

Michael L. Ludensky *Technical Specialist*

EDUCATION:

Dnepropetrovsk University, Ukraine	Ph. D. Environmental Biology 1984
Dnepropetrovsk University, Ukraine	M.S. Biophysics 1974

EMPLOYMENT:

1994 to date Lonza Inc. Contractor - Research Associate – Technical Specialist

1991 - 1994 Marine Biocontrol Corp.	Senior Research Biologist
1989 - 1991 Ukrainian Academy of Sciences	Project Manager
1985 - 1989 Ukrainian Mining Academy	Senior Project Scientist
1974 - 1985 Industrial Water Laboratory	Research Biologist

PRODUCT/TECHNICAL SPECIALTIES:

- Biocides and antimicrobials (treatment programs and mechanisms of action)
- Technical expertise in Zebra Mussel control and mitigation
- Micro- and macrofouling monitoring and risk assessment
- Biofilm growth, monitoring and control in water treatment applications
- Algae control and algicide testing
- Biocides efficacy testing for various applications

TECHNICAL MEMBERSHIPS: ASM, NACE, IBBS

DUTIES AT LONZA:

- Support in commercialization of halohydantoins and quats
- Development and testing of novel biocides
- Analysis of competitive products for WT applications
- Support Lonza biofilm monitoring activities
- Enzyme-based water treatment product line
- Ballast Water Treatment initiative

LONZA PUBLICATIONS AND PRESENTATIONS:

1. Ludensky, M. Microbiological control in cooling water systems" Chapter 5.2 in W. Paulus Directory of Microbicides for the protection of materials. A Handbook. Springer 2005.

2. Ludensky, M., Sweeny, P., Lammering, D., and A. Zakarian. Equinox – novel halogen stabilizer for cooling water systems. AWT 2004.

3. Ludensky, M. Control and monitoring of biofilms in industrial applications. International Biodeterioration and Biodegradation, 2003, 51(4): 255-263.

4. Ludensky, M., Sweeny, P., and F. Himpler. Equinox – new slimicide for papermaking applications. Proceeding of Papermakers Conference, Bratislava, Slovak Republic, 2003.

5. Himpler, F., Sweeny, P., and M. Ludensky. The benefits of a hydantoin-based slimicide in papermaking applications. APPITA, Australia, 2001.

6. Sweeny, P., and M. Ludensky. "Furnish compatibility and efficacy of oxidizing slimicides" TAPPI 2001.

7. Ludensky, M. Control and monitoring of biofilms in industrial applications. Presentation at the 2nd International Symposium "Disinfection and Hygiene – Future Prospects". Wageningen, October 2001. The Netherlands.

8. Coulburn, J., M.Ludensky, D.G. Allison, and P. Gilbert. "Characterization biofilm formation and fouling in paper manufacturing", poster submitted for Symposium "Community structure and Cooperation in Biofilms" of general Meeting of the Society for General Microbiology (UK), Exeter, September 12-15, 2000.

9. Coulburn, J., M.Ludensky, D.G. Allison, and P. Gilbert "Characterization and In-vitro modeling of biofilm formation and fouling of a paper mill", poster presented at ASM Conference on Biofilms 2000. Big Sky, MO, July 16-20, 2000.

10. Ludensky, M., and P. Sweeny. "Biocidal action of oxidizers on biofilm and planktonic systems", presented at SIM / IBBS Meeting, August 1999, Washington, DC.

11. Ludensky, M. "Biofilm monitoring in industrial applications", presented at the Biofilm Club Meeting "Biofilms: the good, the bad and the ugly", September 1999, Gregynog, UK. pp. 81-89.

12. Elsmore, R., Gilbert, P., Ludensky, M., and J. Coulburn. "Biofilms in paper manufacturing", the Biofilm Club Meeting "Biofilms: the good, the bad and the ugly", September 1999, Gregynog, UK. Pp. 129-137.

13. P. Sweeny, M. Ludensky, and O. Borokhov. Mill performance of a brominated methylhalohydantoin slimicide. TAPPI 99 Proceedings, Atlanta,GA.

14. Ludensky, M., Himpler, F., Sweeny, P. "Control of biofilms with cooling water biocides" Corrosion-98 Presentation, Paper 522. San-Diego, CA

15. Ludensky, M. "An automated system for biocide testing on biofilms", J. of Industrial Microbiology. 1998, 20(2):109-115.

16. Ludensky, M., Himpler, F., Sweeny, P. Control of biofilms with cooling water biocides" Materials Performance, 1998, 10:50-55.

17. Ludensky, M., and O. Borokhov. "Biofilm monitoring by ATP bioluminescence techniques", poster presented at ASM-98. Atlanta, GA.

18. Ludensky, M. "Monitoring ATP in Pseudomonas aeruginosa biofilms", poster presented at IAWQ Conference on Biofilm Systems. 1998. Chicago, IL.

19. Ludensky, M. "Biofilm response to non-oxidizing biocides", poster presented at SIM-97. Reno, NV.

20. Ludyanskiy, M.L., and F.J. Himpler. "The effect of halogenated hydantoins on biofilms". Corrosion 97, 1997, Paper 405, NACE.

21. Ludyanskiy, M. "An automated system for biofilm monitoring", presented at the ASM Conference on Microbial Biofilms, 1996, Utah.

22. Ludyanskiy, M.L. and S.J. Colby. "A laboratory method for evaluating biocidal efficacy on biofilms", CTI, 1996, paper TP 96-07.

23. Mills, E.L., G. Rosenberg, A.P. Spidle, M. Ludyanskiy and B. May. "A review of the biology and ecology of the Quagga Mussel (*Dreissena bugensis*), a second species of freshwater Dreissenid introduced to North America", American Zoologist, 1996, 36:271-286.

24. Donskoy, D. and M. Ludyanskiy. "The impact of acoustics on zebra mussel veligers", presented at the Meeting of American Acoustical Society, 1996.

25. Ludyanskiy, M.L. "Long term post-invasion effects of the zebra mussel in European river systems: the Dnieper and Bug River experiences", presented at the New York Sea Grant Workshop "Zebra mussels in large river systems", 1996, Albany, NY.

26. Ludyanskiy, M.L. "*Dreissena polymorpha* in Large European Rivers", presented at the Annual Meeting of the Delaware Section of American Fisheries Society, 1996, Camden, NJ.

27. Mills, E.L., G. Rosenberg, A.P. Spidle, M. Ludyanskiy and B. May. "A review of the biology and ecology of the Quagga Mussel (*Dreissena bugensis*), a second species of freshwater Dreissenid introduced to North America", presented at the Annual Meeting of the American Society of Zoologists, 1995, Missouri.

28. Donskoy, D., and M.L. Ludyanskiy. "Low frequency sound as a control measure for zebra mussel fouling", Proceedings of the 5th International Zebra Mussel and other Aquatic Nuisance Organisms Conference, 1995, Toronto, Canada, p.103-112.

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Exhibit B

5 Fields of application

5.1 Efficacy of biocides against biofilms

SIMONE SCHULTE, JOST WINGENDER and HANS-CURT FLEMMING

5.1.1 Characteristics of biofilms

The preferred form of life of microorganisms occurs in aggregates. In natural, technical or medical environments, pure cultures are the exception rather than the rule. It is assumed that more than 99% of the microorganisms on Earth live in aggregates (Wimpenny, 2000). Phenomenologically, such aggregates can differ widely, ranging from microcolonies or films on surfaces ("biofilms"), flocs ("floating biofilms") to sludge. The scientific community has agreed to subsume these phenomena under the somewhat inexact expression "biofilms". They all have a common feature: the cells live in close associations at high densities and are embedded in an organic matrix of biopolymers, the so-called extracellular polymeric substances (EPS; Wingender et al., 1999a) which are produced by the organisms themselves. In everyday life, they are known as "slime". Biofilms are the first form of life recorded on Earth, dating back over 3.5 billion years (Schopf et al., 1983) and they also are the most successful form of life. They are found even under extreme conditions such as for example a range of pH-value between 0.5 and 14, temperatures from −5 to 120°C, under strong irradiation as present in primary cooling cycles of nuclear power plants, pressure up to 1,000 bar as encountered on the deep sea floor or shear forces as prevailing at the impact point of water falls (examples compiled by Flemming, 1996). Biofilms develop on virtually any surface in natural soil and aquatic environments, on tissues of plants, animals and humans as well as in man-made technical systems (Costerton et al., 1987; Flemming and Schaule, 1996a). Biofilms also develop on medical devices, causing persistent infections in humans (Costerton et al., 1987). Biofilms are commonly attached to a solid surface (substratum) at solid-water interfaces, but they can also be found at water-air and at solid-air interfaces.

Biofilm systems represent accumulations of microorganisms (prokaryotic and eukaryotic unicellular organisms), EPS, multivalent cations, inorganic particles, biogenic material (detritus) as well as colloidal and dissolved compounds. Polysaccharides are characteristic components of the EPS, but proteins, nucleic acids, lipids and humic substances have also been identified, sometimes in substantial amounts (Wingender et al., 1999a). EPS are considered as key components that determine the structural and functional integrity of microbial aggregates. EPS are involved in the formation of a three-dimensional, gel-like, highly hydrated and locally charged (often anionic) biofilm matrix, in which the microorganisms are more or less immobilized. EPS are responsible for binding cells and other particulate materials together (cohesion) and for anchoring biofilms to the substratum (adhesion). They create a microenvironment for sessile cells which is conditioned by the chemical nature of the EPS matrix. In general, the proportion of EPS in biofilms can vary between roughly 50 and 90% of the total organic matter (Christensen and Characklis 1990; Nielsen et al., 1997). Figure 1 shows a conceptual view of a biofilm as reconstructed from horizontal sectioning by confocal laser scanning microscopy (Costerton et al., 1994).

An important aspect of biofilms is their structural and functional heterogeneity with pores and channels. For example, active heterotrophic bacteria may consume oxygen at a rate which is faster than its diffusion rate. In such a case, even in an aerobic system an anerobic zone will develop underneath an aerobic zone and give rise to the development of organisms which would not be expected in an aerobic system. This can lead to the growth of sulphate reducing bacteria at the base of biofilms on the walls of fully aerated water reservoirs (Christensen and Characklis, 1990).

Although the details of biofilm development processes vary according to species general distinct developmental steps have been recognized in bacterial biofilm formation (for review, see O'Toole et al., 2000; to Dunne, Jr., 2002). These include the initial attachment to a surface, followed by the formation of microcolonies, and finally the maturation of microcolonies into an established biofilm, which is maintained in a stable form by the EPS matrix. Environmental parameters (e.g., nutrient availability, osmolarity, pH, oxygen tension, temperature) seem to determine the transition from planktonic life to growth on a surface. According to Donlan and Costerton (2002), bacteria form biofilms preferentially at high-shear locations in natural and industrial systems; thus, planktonic cells can adhere to surfaces and initiate biofilm formation under turbulent flow in the presence of shear forces exceeding Reynolds numbers of 5,000 (Donlan and Costerton, 2002). Mature biofilms are highly viscoelastic, and are stronger and more resistant to mechanical breakage when grown in high-shear environments compared to biofilms formed under low-shear conditions.

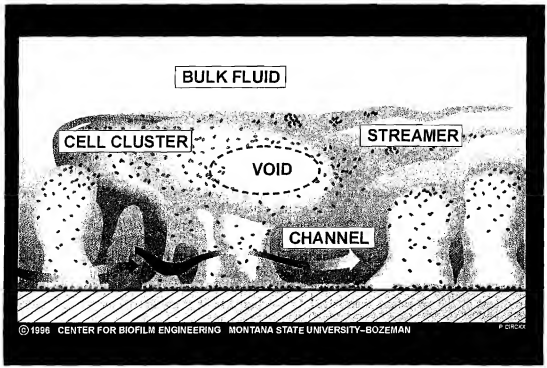

Figure 1 Vertical view of a biofilm (after Costerton et al., 1994).

In recent years, it has become obvious that the biofilm mode of growth is associated with a specific expression of genes and altered growth rates. To take into consideration the adoption of characteristic biofilm phenotypes by planktonic bacteria, a modern definition of a biofilm has been given by Donlan and Costerton (2002), who described a biofilm as "a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of EPS that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription".

Biofilms can have both beneficial and detrimental effects. Beneficial functions include the degradation and turnover of organic matter in natural soil and water environments, or the purification of raw sewage in wastewater treatment plants. Biofilms can have detrimental effects in the human environment when occuring in the wrong place and/or the wrong time. This undesired development of biofilms on surfaces is referred to as *biofouling* (Flemming, 2002). Biofilms can be involved in the destruction of the materials they colonize; these processes are described as *biocorrosion*, *microbially influenced corrosion* (MIC) or *biodeterioration* (Dowling et al., 1991). The damage of biofouling is very difficult to assess, but even crude estimates amount to many millions of $US every year in industrialized countries (Flemming, 1996).

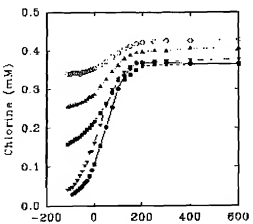

Figure 2 Transient profiles measured at high chlorine concentrations (0.28 to 0.42 mM) in a dual-species biofilm (*P. aeruginosa* and *klebsiella pneumoniae*). The chlorine profiles were recorded 3 (•), 15 (▼), 30 (■), 45 (▲), and 60 (○) min after the start of the chlorine dosing. Zero on the x axis corresponds to the surface of the cell cluster as estimately visually at the start of the experiment. (in DeBeer et al., 1994b).

Biofilms are involved in all kinds of biofouling (Kent, 1988; Flemming, 2002). When biofilms develop on ship hulls, in industrial pipe systems and other fluid flow systems, they increase frictional resistance due to the viscoelastic properties of the EPS matrix. This may lead to a substantial pressure drop and increase in energy consumption or to a reduction of speed of vessels. Christensen and Characklis (1990) assessed that a biofilm of 50 μm thickness, calculated as rigid roughness can lead to a speed loss of 5–12% of a vessel. In cooling water systems, they cause increase in resistance to heat energy transfer, increase in fluid frictional resistance, or acceleration of metallic corrosion. The performance of a heat exchanger can be significantly decreased by a biofilm because only diffusional heat transfer is possible (Characklis, 1990b). Convectional heat transfer as achieved by tangential flow of water across the heat exchanger surface is hampered by the biofilm which then acts as an insulating layer for convection. In some cases, biofilms result not only in the unwanted accumulation of biological material on surfaces, but also promote the precipitation of minerals, especially calcium carbonate. This leads to mixed biological and non-biological deposits (Heath *et al.*, 1992) which are particularly difficult to remove. Calcium precipitation is an important aspect in scaling of surfaces e.g., on heat-exchanger surfaces, on separation membranes, on ship hulls and oil rigs.

In drinking water distribution systems, biofilms provide a habitat for potentially pathogenic microorganisms, which can be released into the water and pose a health hazard to the consumer; they may also be the cause of organoleptic problems. Figure 3 shows a scanning electron micrograph of a biofouling case in which the biofilm developed on a synthetic rubber coating of a valve in a drinking water system, harbouring hygienically relevant organisms such as *Citrobacter* (Kilb et al., 2003). The material between the cells is EPS embedding the biofilm organisms. Due to dewatering, the EPS collapsed but it is still apparent that this material has integrated the bacteria in its matrix.

Contamination of ultrapure water by biofilm bacteria interferes with the production of microchips. In the beverage and food industry, biofilms on equipment surfaces can be involved in the spoilage of the products. Biofilms can also be important in the clinical setting, when they develop on medical devices (Donlan, 2001) such as implants, catheters, contact lenses, leading to life-threatening infections.

The above-mentioned examples show clearly that many problems in technical water systems are caused by biofilms and not by planktonic cells. Thus, countermeasures against biofouling must be directed against suface-attached biofilms. These measures should include the detection, monitoring, removal, prevention or at least control of biofilm formation (Flemming, 2000). A variety of sanitation measures for the treatment of biofouling exist such as regular cleaning using physical methods (e. g., rinsing, brushing, ultrasonic treatment),

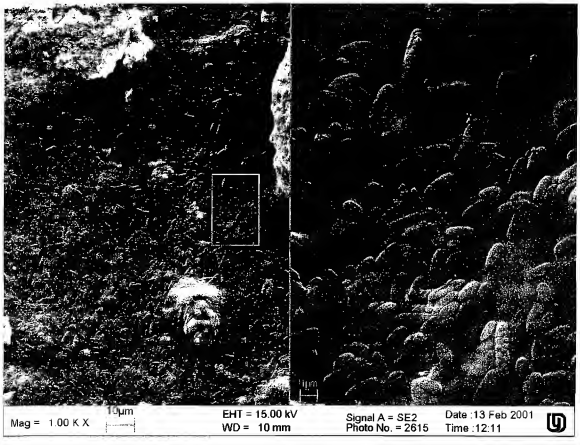

Figure 3 SEM micrograph of a biofilm on a synthetic rubber coating of a drinking water valve (from Kilb et al., 2003).

application of chemical agents (oxidants, alkali, surfactants, enzymes, complexing substances, dispersants) to kill and detach biofilm organisms, limitation of nutrients to minimize microbial growth, etc. (Flemming and Schaule, 1996b; Flemming, 2002).

As indicated in these examples, unwanted biofilms occur in very different fields and are approached rather individually. In most cases, there is one common misunderstanding: the problems are considered as a kind of "technical disease" in terms of a medical analogy which is reflected in the use of the medically defined term "disinfection". While the killing of invading microorganisms will be the cure of choice in living organisms, this is in most cases not the case for technical systems. In contrary to an immune system, which eliminates dead microorganisms, dead biomass in a technical system will stay in place and provide nutrients for cells which enter the system later. Therefore, it is most important to distinguish between killing and cleaning, with cleaning being at least equally important as killing. Some biocides may have an additional cleaning effect but most do not. In such cases, a disinfection will not lead to a solution of the problems but sometimes lead to rapid regrowth and a "saw-toothed curve" of biofilm development with a rising baseline (Flemming, 1991). This is frequently observed in practice and represents the cause of failures of anti-fouling measures using disinfectants and biocides, but it is usually not published. Highlighted by this background, we will separately address biocidal vs. cleaning effects in the following discussion of various biocides.

5.1.2 Definitions of antimicrobial agents

It is useful to define the different terms concerning antimicrobial agents applied to treat biofouling problems.

A *biocide* is a chemical agent that inactivates living organisms, pathogenic and nonpathogenic. In the context of the application of biocides as antifouling agents, Cloete et al. (1998) described biocides as "antimicrobial agents employed in various spheres of human activity to prevent, inhibit or eliminate microbial growth". In the control of biofouling, the primary aim of biocide application is usually to reduce microbial numbers on a surface in order to restore or maintain the proper functioning of a technical system. A wide variety of oxidizing and non-oxidizing inorganic and organic biocides are known; they are commonly used in commercial formulations to control biofouling in industrial systems or in clinical environments. A number of biocides can also have a cleaning function; in this context, cleaning means the physical removal of unwanted biofilm material from a surface.

Disinfectants and antiseptics are biocides or products that are primarily used to inhibit or destroy hygienically relevant microorganisms; they are used to prevent infection, i.e., transmission of pathogenic or potentially pathogenic microorganisms. As for the treatment of surface-attached microorganisms, disinfectants are applied to inanimate objects or surfaces, whereas antiseptics are used to inactivate microorganisms in or on living tissues (Block, 1991a; McDonnell and Russell, 1999). In biofouling treament, biocides can be used as disinfectants, when the aim is to selectively inactivate disease-causing microorganisms, for example *Legionella* bacteria in biofilms of cooling water systems and hot water systems, or opportunistic microorganisms on the surfaces of medical devices.

Antibiotics [II,20.11]* are naturally occurring organic substances produced by certain microorganisms (bacteria and fungi), which inhibit the growth of other organisms, generally at relatively low concentrations. Antibiotics do not play an important role in their application in technical systems, but are used therapeutically to inactivate planktonic and biofilm organisms in the treatment of infectious diseases.

In general, biocides have a broad spectrum of activity and have multiple targets; an exception are antibiotics, which tend to have specific cellular targets. The response of microorganisms to biocides depends on the type of organism, the biocide itself, and the concentration. It is not easy to elucidate the exact mechanism of action of a biocide. The reason for this is that more than one cell constituent may be affected, and consequently the problem is to distinguish the primary effect from the secondary effects, which may, however, contribute to cell death. Target structures of biocides include the cell wall, cytoplasmic membrane and ribosomes of vegetative cells, the coat and cortex of bacterial spores, and the envelope and capsid of viruses; biocides can react with macromolecules such as proteins (structural proteins, enzymes), nucleic acids and polysaccharides. The results are the disrupture of membranes with leakage of intracellular components, and the destruction of many cellular functions, including replication, transcription, protein synthesis, and general metabolism. A number of recent reviews give more detailed informations about the mechanisms of action of biocides (Cloete et al., 1998; McDonnell and Russell, 1999). See also Part One, Chapter 2.

*see Part Two – Microbicide Data

5.1.3 Resistance of biofilm organisms to biocides

Biofilms are known to exert enhanced resistance to biocides (LeChevallier et al., 1988 a,,b). There are different mechanisms of resistance, depending upon the biocide, the biofilm and the environmental conditions. Some of the major factors are discussed in the following sections.

5.1.3.1 Influence of abiotic factors

In adverse environments, microorganisms in biofilms are supposed to have a survival advantage over planktonic cells (Marshall, 1985). Under practical conditions, the success of biocide treatment depends not only on the biological resistance properties of the microorganisms *per se*, but is also determined by other factors such as choice and type of biocide, treatment regime, and a number of different environmental parameters (Cloete and Brözel, 2002). Since several of these factors can interact antagonistically or synergistically, it cannot always be determined unequivocally to which extent the resistance properties of one organism actually contribute to their survival.

The influence of abiotic factors on biocide efficacy has been largely studied on planktonic populations (Bessems, 1998), but can be expected to be also relevant to biofilms. For example, the enhanced efficacy of many biocides with increasing *temperature* has been described for the treatment of *Pseudomonas aeruginosa* biofilms; thus, a formulation of peracetic acid and hydrogen peroxide caused an increase in killing when the temperature was increased within the temperature range of 10°C to 50°C (Blanchard et al., 1998).

The *incorporation of abiotic particles* such as kaolin, calcium carbonate or iron-containing corrosion products into biofilms can result in a reduced biocide efficacy as has been shown for chlorine and monochloramine (LeChevallier, 1991; Srinivasan et al., 1995). This effect has important implications, since biofilms can be dominated by particulate matter in industrial systems such as cooling towers or water pipelines.

In most practical situations, biofouling is caused by submerged biofilms. Therefore, *hydrodynamic conditions* have to be taken into consideration for biocide application. Flow velocity can have a marked effect on the killing and detachment of biofilms by biocides in aquatic systems. For example, Bott (1998) mentioned that, after 1 hour in the presence of a chlorine concentration of 4.7 mg/L, the removal of an established biofilm was approximately 20% higher at a flow velocity of 1.27 m/s compared to a flow velocity of 0.86 m/s. Turbulent flow improved the inactivation of sessile bacteria (*P. fluorescens*) on metal coupons by 0.15 mg/L ozone (contact time of 1 hour), resulting in a decrease of about one order of magnitude in cell density compared to the killing of the same biofilms under static conditions (Viera et al., 1999a, b). The transition from laminar to turbulent flow was accompanied by a 99.7% increase in the inactivation of biofilm bacteria (*P. aeruginosa*) during treatment with 15 mg/L peracetic acid (Blanchard et al., 1998). Possible causes for enhanced biocidal efficacy under high flow rates are thought to be improved mass transfer and/or a higher shear at the biofilm/water interface, promoting the access of the biocide into the biofilm.

5.1.3.2 Enhanced resistance of biofilms

In practical situations, biofilms are often difficult to eradicate and prove recalcitrant to the application of biocides. According to Gilbert et al. (2001) biofilms are 10 to 1 000 times less susceptible towards a wide variety of different antimicrobial agents than are the corresponding planktonic cells. This phenomenon is supposed to be due essentially to various resistance mechanisms that are associated with the biofilm mode of growth. Recent reviews have been published, covering different aspects of resistance of biofilms to antimicrobial agents (Brown and Gilbert, 1993; Morton et al., 1998; McDonnell and Russell, 1999; Gilbert et al., 2001; Lewis, 2001; Mah and O'Toole, 2001). In many cases the exact mechanisms of biofilm resistance are still unclear and are only beginning to be elucidated. Here, a short summary is given on the known resistance mechanisms of established biofilms that undergo treatment with biocides.

In the context of this chapter, resistance is defined as the ability of a microorganism to grow in the presence of elevated levels of an antimicrobial substance or to survive the treatment with an antimicrobial substance. Under practical conditions, elevated levels of a biocide are those which are higher than the concentrations usually used for the killing or control of microbial contaminations in a specific application. It must be pointed out that most biofilm susceptibility studies in the laboratory and in practical situations have been performed on already established biofilms, analyzing the survival and persistence of biofilm cells, while the efficacy of biocides on biofilm growth is rarely considered. As to inhibition of growing cells, biofilm organisms have been described which do not grow better than planktonic cells in the presence of many antimicrobials (Lewis, 2001), indicating that biocides may prevent or at least delay and control the colonization and biofilm formation on surfaces. However, mature biofilms exhibit an increased resistance to killing by biocides.

Two major types of microbial resistance can be distinguished: intrinsic and acquired resistance. Intrinsic (innate) resistance refers to a natural chromosomally controlled property, including physiological adaptation,

that is specific for a certain type of microorganism. Acquired resistance may be due to mutations with subsequent selection of resistant mutants from the population which has been exposed to the biocide, or it may result from the uptake of plasmids or transposons which confer resistance to biocides (Morton, 1998; McDonnell and Russell, 1999). Formation of a biofilm can be regarded as a physiological (phenotypic) adaptation, and thus represents an intrinsic mechanism of microbial resistance to biocides. At present, it is not known if acquired resistance is of importance in biofilm resistance. It can be speculated that the high cell densities in biofilms may enhance the probability of spontaneously resistant mutants to be selected on exposure to sublethal concentrations of biocides; in addition, high cell numbers may promote horizontal transfer of genes expressing resistance to biocides (Davey and O'Toole, 2000).

Many different mechanisms of biofilm resistance are discussed in the literature, reflecting the different ways of biofilm organisms to withstand biocides. These mechanisms include physical and chemical diffusion-reaction barriers in the biofilm restricting biocide penetration of the biofilm, slow growth rate of biofilm cells due to nutrient limitation, activation of general stress response genes, the emergence of a biofilm-specific phenotype, and the presence of persister cells.

5.1.3.3 Transport limitation by reaction-diffusion interaction

One of the first explanations for the increased resistance of biofilm organisms to biocides was diffusion limitation (Costerton et al., 1987). The reported resistance often refers to an increased resistance of the established biofilm population as a whole to the killing of the applied biocide. However, when biofilm bacteria are dispersed (removed from the intact structure of the biofilm), the susceptibility of these suspended organisms may be enhanced or equal to that of planktonically grown cells. This effect has been reported for biofilm bacteria treated with chlorine and chlorosulfamate (Gilbert et al., 2001). Griebe et al. (1994) found that homogenized *P. aeruginosa* biofilms were significantly more sensitive to chlorine compared to intact biofilms, whereas monochloramine showed decreased killing against homogenized biofilms. When biofilms of *P. aeruginosa* were dispersed, their sensitivity to quaternary ammonium compounds was strongly enhanced, almost to the level of planktonically grown cells; however, recovery of sensitivity was poor in suspended biofilms of *Staphylococcus aureus* (Campanac et al., 2002).

These observations indicate that, depending on the type of biocide and species-specific biofilm formation, the resistance of single biofilm cells within the bulk biofilm population is not necessarily changed compared to planktonic cells. In these cases, resistance of the biofilm population is brought about by the characteristic spatial arrangement of cells within the EPS matrix inherent to the biofilm mode of growth. Since the development of a three-dimensional biofilm structure depends on the presence of EPS, the production of these molecules can be considered as part of a physiological adaptation process of the whole population during biofilm formation with the consequence of enhanced resistance to antimicrobial treatments (Hentzer et al., 2001).

It has been suggested that the EPS matrix presents a potential barrier which delays or prevents biocides from penetration into the biofilm and from reaching target organisms in all parts of a biofilm. The plausible assumption was that mass transfer of antimicrobial agents to microorganisms might be restricted by the EPS matrix, acting as a diffusion barrier or by interaction of the biocide with matrix components. However, no significant diffusion limitation was observed for small, non-reacting molecules the size of biocides. They were shown to diffuse freely in the biofilm matrix and to penetrate biofilms of up to 1 mm in thickness relatively quickly, within seconds or minutes (Stewart, 1996, 1998). In addition, biofilms have been shown to be structurally heterogeneous with pores and channels, allowing for convective flow to a small extent throughout the biofilm, so that access of biocides is not necessarily limited to most parts of a biofilm.

Another explanation for reduced biocide penetration into biofilms is the interaction between biocide and biofilm constituents, including cells and EPS; a result would be depletion of the antimicrobial compound in the biofilm interior. The underlying mechanisms may be chemical reactions of the biocide with, or sorption to, the biofilm components or the enzymatic degradation of the biocide, resulting in a restricted penetration of the biocide into the biofilms (reaction-diffusion interaction mechanism).

A large number of studies has been performed on the chemical interaction between biofilms and oxidizing biocides [II, 21] such as chlorine (sodium hypochlorite) and hydrogen peroxide, which have wide-spread application in industrial and clinical settings. Using a chlorine-sensitive microelectrode, chlorine penetration into biofilms containing *P. aeruginosa* and *Klebsiella pneumoniae* was found to be a function of simultaneous diffusion and reaction in the biofilm matrix (de Beer et al., 1994b; Figure 2); chlorine concentrations were only 20% or less of the concentration in the bulk liquid over a 1 to 2 hour period. The shape of the chlorine profiles indicated chlorine consumption within the biofilm matrix. Similar results were obtained using an artificial biofilm system consisting of *P. aeruginosa* entrapped in alginate and agarose gel beads (Xu et al., 1996).

EPS are considered as reactive compounds for biocides. However, this depends on the nature of the antimicrobial agent and the composition of the EPS, and whether the EPS matrix constitutes a penetration barrier and protective structure for biofilm organisms. For example, in mucoid strains of *P. aeruginosa*, slime formation afforded protection against chlorine, but not against hydrogen peroxide (Wingender et al., 1999b). It was

demonstrated that chlorine chemically reacted with the exopolysaccharide alginate (major EPS component of mucoid *P. aeruginosa*) under rapid chlorine consumption and release of trihalomethanes; in contrast, hydrogen peroxide did not interact with the alginate (Wingender et al., 1999b).

Aldehydes [II, 2.] react with amino acid residues of proteins, which may be a substantial fraction of the EPS. The reaction of glutaraldehyde with extracellular proteins in *P. fluorescens* biofilms was supposed to reduce the antimicrobial action of the biocide (Pereira and Vieira, 2001).

Cationic biocides may be immobilized by binding to negatively charged microbial exopolysaccharides; thus, cationic biocides including biguanides [II, 18.3.] and quaternary ammonium compounds [II, 18.1.] interact with EPS molecules by electrostatic interactions (Morton et al., 1998), restricting permeation of the biocide through the biofilm. This mechanism may also apply to certain antibiotics; for example, their interaction with the polyanionic exopolysaccharide alginate has been supposed to contribute to retarded penetration into thick biofilms (Ishida et al., 1998).

Biofilm organisms produce a large number of different degradative enzymes which accumulate in the biofilm matrix (Wingender and Jaeger, 2002) and may provide a potential for the enzymatic degradation of biocides. For example, catalase enzymes neutralize hydrogen peroxide by disproportionation of the biocide into oxygen and water. In biofilms of *P. aeruginosa*, a constitutively expressed catalase (KatA) has been shown to protect the bacteria by preventing full penetration of hydrogen peroxide into the biofilms (Stewart et al., 2000). A second catalase (KatB) was induced in biofilms as an adaptive response to sublethal amounts of hydrogen peroxide; this effect was supposed to be an acquired resistance mechanism for biofilm protection when initial biocide levels were sublethal (Elkins et al., 1999). Additional evidence for the importance of catalases in the protection of biofilm-forming bacteria comes from the observation that catalase-deficient mutants revealed enhanced susceptibility to hydrogen peroxide (Elkins et al., 1999; Wingender et al., 1999b). Another example of enzymatic inactivation is the degradation of some antibiotics by β-lactamase enzymes in bacterial biofilms (Giwercman et al., 1991). Calculation of β-lactam penetration reveals that this catalytic reaction can lead to severe antibiotic penetration failure (Stewart, 1996).

All of these observations indicate that biofilm constituents in the outer layer of biofilms can interact with certain biocides and remove them in such quantities and at sufficiently high rate to protect more deeply embedded microorganisms (Xu et al., 1996). Practical consequences are that complete inactivation of biofilm organisms can only be expected if large enough quantities of biocides are applied over a sufficiently long period of time or even continuously, ensuring complete penetration of the biofilm. Subinhibitory levels of biocides may induce the formation of EPS components (Leyval et al., 1984; Elkins et al., 1999), which will be involved in enhanced survival due to the reaction-diffusion interaction mentioned above. However, the reaction-diffusion limitation mechanism based on interactions beween biocide and EPS components cannot always completely account for biofilm resistance, and other physiological mechanisms must be expected to be implicated in resistance.

5.1.3.4 Slow growth rate and general stress response

In many biofilm environments, microorganisms live under conditions of nutrient limitation (Marshall, 1985). Biofilms preferentially grow in oligotrophic conditions, so that supply of nutrients may be limiting; competition for nutrients among biofilm organisms which are typically present at high cell densities may further promote nutrient limitation. In addition, nutrient access to all parts of a biofilm may be incomplete due to consumption of the biocide. This is supposed to be one reason for the spatial heterogeneity of physiological activity in biofilms (Huang et al., 1995; Xu et al., 2000; Mah and O'Toole, 2001).

Growth rate has been implicated in susceptibility to biocides. At slow growth rates, susceptibility to certain antimicrobial agents may be diminished. Distinct regions of faster and slower growth have been observed throughout the same biofilm (Wentland et al., 1996). Bacteria located deep in the biofilm may experience a nutrient-deficient environment, so that in these parts of the biofilm bacteria grow slowly or not at all (Huang et al., 1995). Nutrient limitation may switch cells into a dormant and thus protected phenotypic state. A consequence of nutrient gradients is the development of physiological heterogeneity within a biofilm, which is also reflected in a differential response of individual cells within the same biofilm. For example, the pattern of respiratory activity in a *K. pneumoniae* biofilm exposed to monochloramine showed that the bacteria near the biofilm-bulk liquid interface lost activity first (Huang et al., 1995). Many antibiotics only kill growing cells or are more effective against rapidly dividing cells, so that absence or decrease of growth may be a reason for biofilm resistance to these types of antibiotics. In this respect, biofilms resemble stationary-phase planktonic cells in batch cultures, which are also characterized by reduced growth and are less susceptible to antimicrobial agents compared to planktonically grown logarithmic cells (Spoering and Lewis, 2001).

Based on these observations, it can be expected that the efficacy of biocides can vary greatly, depending on the location and the physiological state of the target cells within a biofilm.

As pointed out, slow growth seems to be responsible for a certain level of resistance, but there is evidence that it only adds protection in addition to other mechanisms (Mah and O'Toole, 2001). It has been suggested that

slow growth is only one aspect of a general stress response triggered by microbial growth within a biofilm. The result would be physiological changes, which can render biofilms more resistant to various environmental stresses, including heat and cold shock, pH changes or the presence of antimicrobial agents. The basis for stress-resistant phenotypes are changes in gene expression and regulation. For example, there is some evidence that alternate sigma factors have a role in biofilm resistance to certain oxidative biocides. Thus, in thin biofilms of *P. aeruginosa*, the sigma factors RpoS and AlgT were shown to be involved in resistance to hydrogen peroxide, but not to monochloramine (Cochran et al., 2000); in thick biofilms, the sigma factors did not contibute significantly to hydrogen peroxide resistance, suggesting a transient role of the sigma factors in this biofilm system.

5.1.3.5 Role of biofilm-specific phenotype

Evidence is accumulating that the the process of attachment to surfaces and growth in a biofilms is associated with the activation and repression of genes, resulting in a biofilm-specific phenotype of the microorganisms within a biofilm community. It is assumed that this process includes the expression of a biocide-resistant phenotype in all or a subset of the biofilm cells (Mah and O'Toole, 2001). Induction of this phenotype may be caused by nutrient limitation, environmental stress, exposure to sublethal amounts of biocides, high cell density or a combination of these factors.

Induction or upregulation of exopolysaccharide production is a phenotypic characteristic of surface-attached bacteria; for example, in *P. aeruginosa*, the transcription of key genes involved in the biosynthesis of alginate is induced soon after the bacteria attach to a solid surface (Davies, 1999). These regulatory processes may contribute to biocide resistance, indirectly by mediating the development of the three-dimensional biofilm architecture and providing protected niches for biofilm organisms, or directly by representing target molecules for the interaction and quenching of biocides as described above.

Biofilm formation is influenced by the phenomenon of quorum sensing, which is the regulation of gene expression-based cell-cell communication, frequently in response to population density (for recent review, see Miller and Bassler, 2001). This kind of bacterial communication is mediated by low-molecular-weight diffusible signal molecules called autoinducers. Among them, N-acylhomoserine lactones (AHLs) are the best studied class of signal molecules. Extracellular accumulation of AHLs above a critical threshold level results in transcriptional activation of a range of different genes with concomitant expression of new phenotypes. Thus, it can be expected that quorum sensing may influence biocide resistance, either indirectly by influencing the formation of the biofilm (Davies et al., 1998), or by regulating genes whose products are directly involved in resistance.

Other possible phenotypic changes that are discussed include decrease of membrane permeability due to alterations in membrane compositions or the upregulation of multidrug efflux pumps that could extrude biocide molecules from the cell interior (Gilbert et al., 2001; Mah and O'Toole, 2001). However, additional studies are still necessary to elucidate the relevance of these mechanisms for biofilm resistance to biocides.

5.1.3.6 Role of persister cells

Recently, an alternate hypothesis was suggested to explain biofilm resistance to killing based mainly on studies of the efficacy of various antibiotics against biofilms. It is assumed that, similarly to multicellular organisms, damaged cells undergo a programmed cell death, while a small population of cells (persisters), which are defective in their suicide response, would survive the exposure to the antimicrobial agent in protected niches within the biofilm (Lewis, 2000, 2001). These persisters would benefit from defective biofilm organisms through nutrient provision and biocide quenching. Thus, biocide-induced damage triggers cell death and would eliminate defective cells within the biofilm population. When biocide treatment is discontinued, the persisters would start to multiply, producing a new biofilm population consisting mostly of biocide-sensitive cells and again only a minor fraction of new persisters. In contrast to induction processes and genetic mechanisms necessary for survival, persisters can react immediately and survive a sudden challenge by a biocide. Persisters are not mutants but arise at a considerably higher rate (10- to 10 000-fold) than mutants (Lewis, 2000). It was emphasized that dense populations of either stationary-phase cells or biofilms favors persister formation (Spoering and Lewis, 2001); this phenomenon was supposed to explain similar resistance of biofilm and stationary phase planktonic cells of *P. aeruginosa* to antibiotics, which were considerably more resistant than logarithmic planktonic cells. In a recent study, antibiotic-resistant variants of *P. aeruginosa* with an enhanced ability to form biofilms were shown to arise at high frequency in response to antibiotic treatment (Drenkard and Ausubel, 2002). It was speculated that some bacteria within a population undergo transient phenotypic changes to antibiotic-resistant variants, which are selected inside mature biofilms by antibiotic treatment; they switch back to the antibiotic-susceptible forms after antibiotic treatment.

These observations indicate that biofilm populations may always contain a sub-set of organisms which ensure survival of the species by the ability to adopt transiently a biocide-resistant phenotype.

5.1.4 Methods to study antimicrobial action on biofilms

In general, three methods can be applied to study the antimicrobial action of biocides against biofilms:

- Planktonic assays
- Dried surface assays (contaminated surfaces)
- Biofilm assays

5.1.4.1 Planktonic assays

The simplest way to investigate biofilm organisms is to remove and suspend them and to carry out further investigations with biofilm suspensions. However, highlighted by the background of observations presented earlier, suspended biofilm organisms are not biofilms any more. The difference orginates from the disruption of the matrix, such that the extrapolation of results of such investigations to real biofilms was to be considered with great scepticism.

The only advantage of this approach is that test systems based on suspended bacteria are well standardized. The test organisms are cultivated in liquid nutrient media, subcultured in fresh media, harvested, washed, and resuspended in a buffer at a defined cell density. The test suspension is then brought into contact with the biocide. After a given contact time, a neutralising medium is used to inactivate the biocide, which is followed by determination of the number of surviving cells. This usually consists of determining the colony-forming units on nutrient-rich agar medium. The procedure represents a traditional and widely used method for determination of biocide efficacy. A series of recommendations, guidelines and "standards" for such purposes are available; these endeavour to standardise the procedures for tests of this type. However, results obtained from experiments with suspended biofilms have to be treated with great scepticism.

5.1.4.2 Surface contamination experiments

Surface contamination experiments are especially relevant in medicine and in the food industry, in terms of disinfection of instruments and work surfaces. They refer to microorganisms adhering to surfaces without physiological formation of further developed biofilms. However, they can be considered as microbial aggregates and differ from suspended organisms. For these applications, there are a series of standardised procedures at national levels, e.g. Germany (DGHM, 1991), France (AFNOR, 1988), Belgium (Reybrouck, 1990), The Netherlands (van Klingeren,1978), and at the European level (CEN/TC 216, 1998). In contaminant-loading experiments, bacterial cells or spores are distributed on a surface, and then fixed to the surface by means of air- or vacuum-drying.

Carrier tests with dried cells on surfaces are not suitable for the evaluation of biocide efficacy towards biofilms. In general, microbial cells dried on carriers are less susceptible to biocides compared to planktonic cells; however, established biofilms grown on surfaces usually display enhanced resistance compared to organisms simply dried on carriers (e.g., Samrakandi et al., 1994; Ntsama.Essomba et al., 1997). A possible reason may be physiological changes which are associated with biofilm formation, and can result in enhanced resistance to biocides. These processes are not involved in carrier tests.

5.1.4.3 Biofilm experiments

There are currently no recommendations or guidelines concerning the standardized cultivation of biofilms; methods which give indications of practical efficacy are the most useful. Attempts have been made to unify procedures for determining the efficacy of biocides; however, the findings of the available literature are difficult or impossible to compare. In principle, this can be described under several headings with respect to the duration of the investigations:

- Cultivation of biofilms
- Biocide treatment
- Removal and homogenisation of the biofilm cells
- Determination of biocide efficacy

5.1.4.3.1 Cultivation of biofilms. The majority of biofilm studies are based on monocultures (Baldry, 1983; Christensen et al., 1990; Samrakandi et al., 1994; Johnston and Jones, 1995; Wood et al., 1996; Ntsama-Essomba et al., 1997; Blanchard et al., 1998; Härkönen et al., 1999; Lindsay and von Holy, 1999; Cochran et al., 2000;

Herruzo-Cabrera, 2000; Bredholt et al., 2001; Spoering and Lewis, 2001). The obvious advantage is that the organisms are well defined; the obvious disadvantage is their lack of representativity to environmental conditions. Defined mixed cultures are rarely employed (Alasri et al., 1992, Fatemi and Frank, 1999). The advantage of biocide studies with monocultures or defined mixed cultures lies in the better reproducibility of the experiment. In this way, variations in the experimental parameters can be chosen or kept constant.

Only in few publications is the action of biocides described for aqueous systems with undefined biofilms with mixed composition (Exner et al., 1987; Mathieu et al., 1990; Goroncy-Bermes and Gerresheim, 1996; Morin, 2000; Holtmann and Sell, 2001; Walker et al., 2001). The advantage of efficacy testing on natural biofilms lies in the greater relevance to the practical situation.

Vastly different methods are available for cultivation of biofilms. Basically, these can be described as either batch-mode or continuous mode methods. In batch studies, growth substrata in the form of coupons or glass slides are placed in e.g. Petri-dishes or other holders filled with medium. Under the action of undefined shear forces, investigations in so-called "beaker" reactors are conducted. A further test system which operates in batch mode and continues to become prominent is the miniaturized test system comprising microtitre plates, in which 96 wells enable the simulation of various experimental conditions simultaneously under static conditions (Geneveaux et al., 1996; O'Toole et al., 1999). In general, biocide tests in batch systems are simpler to run, have shorter durations, and are very simple to carry out. Therefore, they are well-suited for initial comparisons or screenings of different biocides, or different concentrations and contact times of a particular biocide.

Should the biocide require practice-orientated investigation and testing under defined conditions, continuous systems are best suited. The simplest continuous test sytem comprises a flow-through tube (preferably silicon which supports biofilm growth by release of softeners) which acts as a growth substratum (Exner et al., 1987; Mathieu et al., 1990). Equally uncomplicated is the exposure of test surfaces in continuous systems, such as the continuous-flow stirred-tank reactor (CSTR), which consists of a simple glass beaker through which medium is pumped continuously (Hamilton, 2002). The inoculation of biofilm reactors usually is considered as the beginning of the experiment. Often, the reactor is operated in batch mode for 24 hours to enable adaptation of the bacteria before the supply of medium is started. As a rule, the flow velocity is set such that biofilm cell growth is faster than suspended cell growth (Characklis, 1990). In this way, suspended bacteria are rinsed from the reactor.

After defined exposure times, the test substrata can be removed from the system and treated with biocide, or they remain in the system and are analysed at the end of the experiment to determine biocide efficacy. Other, new variations of the CSTR can be suitable as test systems. These include the Calgary Biofilm Device, which utilises shaken microtitre plates (Ceri et al., 1999; Ceri et al., 2001), the flow-cell (Stoodley et al., 2001), the artificial biofilm system (Harkonen et al., (1999), the colony biofilm system (Anderl et al., 2000) and the drip flow reactor (Xu et al., 1998).

More complex reactor systems for biofilm growth under defined conditions, i.e. controlled hydrodynamic conditions with the possibility of subsequent biocide treatment, include the RotoTorque™ (rotating annular reactor) (Characklis, 1990), Robbins Device (McCoy et al., 1981), constant-depth film fermenters (Atkinson and Fowler, 1974; Peters and Wimpenny, 1988, 1989) or the radial flow (Fowler and McKay, 1980) and rotating disc reactors (Loeb et al., 1984). An introduction to these reactor systems is given by Gilbert and Allison (1993).

Rotating annular reactors are used in many water installations to analyse the mechanisms of biocide action. Investigations concerning the action of monochloramine and free chlorine on monoculture biofilms of *P. aeruginosa* were described by Chen et al. (1993a and b), Griebe et al. (1994) and Srinivasan et al. (1995). Christensen et al. (1990) employed a miniaturised rotating annular reactor to investigate the action of hydrogen peroxide in combination with Fe^{2+} on monoculture biofilms of various *Pseudomonas* species.

5.1.4.3.2 Biocide treatment. After establishment of the biofilm, biocide treatment is accomplished by exposure of test surfaces with attached biofilm to the biocide-containing solution. The biocide treatment is performed either by direct application of the biocide to the system in which the biofilm has become established, or separately, following removal of the growth substrata with the attached biofilm. In continuous systems, it is also possible to administer the biocide-containing solution to the entire system, and then rinse after the desired contact time. In this way, intermittent biocide treatment can be accomplished, as can investigations of the subsequent growth of the biofilm following a biocide treatment (recontamination). As in the case of the suspension tests, the procedure is followed by neutralisation of the biocide.

In the case of hydrogen peroxide, catalase is commonly chosen as the neutralising solution, since it cleaves the active agent into water and oxygen. If the biofilm is treated with chlorine, sodium thiosulphate is generally used as neutralising agent. Peroxidase stops the reaction in the case of peracetic acid, while silver compounds are inactivated with sodium thioglycolate or ascorbic acid. If more than one biocide is tested, the neutralisation solution contains a cocktail of the inhibitors, or a universal quenching agent (UQA), the latter of which generally includes peptone, Tween 80, sodium thiosulphate, catalase, and lecithin in deionised water (Lambert et al., 1998; Lambert and Ouderaa, 1999).

5.1.4.3.3 Removal and homogenisation of the biofilm cells. For the quantification of the effect of biocides, in most cases, the biofilm has to be removed from the test surface as it is difficult to quantify biofilms directly. The problem is the fact that bacteria which have been damaged previously by exposure to biocide are completely inactivated by a further, usually mechanical stress. As a consequence of careless removal and homogenisation, the action of a biocidal substance can be overestimated.

A widely used method for removal and simultaneous homogenisation of biofilms consists of treatment with ultrasound. As additional possibilities for removal of biofilm organisms, various methods are used to scrape the colonisation substrata. Depending on the composition of the test surface, rubber scrapers or razor blades can be suitable for complete removal of the biofilm. The removed bacteria can then be transferred to a defined volume of suspension medium. Susbsequently, they are homogenised mechanically. Another method is removal of the biofilm with a sterile cotton swab, which is then transferred in its entirety to a test tube containing a suspension medium. After a defined homogenisation time (e.g., shaking), the swab is removed, leaving the suspended bacteria in the solution.

In test systems which are difficult to access, e.g., tubes, catheters or pipes, quartz sand is commonly used to remove biofilm. Approximately one-third of the respective device is filled with quartz sand of a suitable grain size (diameter of 0.5 to several millimetres), and the remainder is filled with suspension medium. By a particular agitation method, such as rotation of the test surface, the biofilm is abrasively removed and, together with the quartz sand, transferred to a sterile container. The quartz sand is removed by centrifugation, and the supernatant is shaken to homogenise the suspended bacteria.

5.1.4.3.4 Determination of biocide efficacy. As described previously, two basic aspects should be considered concerning the action of biocides:

1. the killing efficacy and,
2. the cleaning efficacy of the biocide.

The action of antimicrobial substances, from inhibition of metabolic activity to physical destruction of the microorganisms, is very complex and difficult to identify, in that microorganisms represent "multiple targets". This means that, in general, introduction of an antimicrobial agent affects more than one cellular component. Therefore, it is difficult to differentiate between primary and secondary effects. Through judicious choice of different methods, it can be investigated with some selectivity whether the biocide inhibits metabolic activity, if particular enzymes are inactivated, and/or if destruction of the cell wall, respiratory apparatus, ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) are involved. By a combination of the following detection methods, conclusions can be made concerning the sites at which a biocide is active.

The number of surviving bacteria is generally measured by culture-based methods which are related to the surface colonising area following removal of the biofilm bacteria from the surface. Thereby, all bacteria which are still capable of multiplication under particular conditions (medium composition, incubation temperature/time) are considered. These methods commonly do not reflect the real situation in a biofilm because in this case, many organisms show activity but cannot be cultivated (viable but non culturable, "VBNC", Kell et al., 1998). Thus, the numbers of viable organisms measured by traditional cultivation-based methods are usually grossly underestimated. Certainly, a considerable advantage of these methods is that they have been employed for a long time and therefore, even when the measured colony counts do not reflect the actual cell number, they are process-relevant and give some practical measure of the microbial burden in a system.

A somewhat modified cultivation-based method, which allows the metabolic activity of surface-attached bacteria to be recognised, consists of coating test coupons in Petri dishes with a layer of agar, followed by overnight incubation. The agar is then removed from the surface and incubated further with the contact-side facing up. The metabolically active bacteria form colonies which can be counted (Bredholt et al., 1999).

A further possibility to determine the active cells in biofilms is the application of fluorescent dyes. The advantage of these methods is the short incubation time. Within a few hours, the required information is obtained, whereas cultivation-based methods can require days or even weeks. The determination of cell showing respiratory activity is performed with e.g., the tetrazolium salt 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Schaule et al., 1993). Another microscope-based detection method for active and inactive bacteria involves staining with the fluorochromes contained in the Live/Dead *Bac*Light Bacterial Viability Kit. This comprises two fluorochromes, both of which bind to DNA. The differentiation between "living" and "dead" cells is based on membrane integrity. Also, the ATP content can be measured with the surface monitoring kit 1243-114; this is a measure of the metabolic activity of biocide-treated bacteria (Bredholt et al., 1999).

It is also possible to measure the vitality of biofilm bacteria through measurement of the transmembrane potential with Rhodamine 123 or $DiBAC_4(3)$ (Comas and Vives-Rego., 1997; Lisle et al. 1999).

The ability of biocide-treated biofilms to utilise nutritional substrates can be determined by a "direct viable count" (DVC), which is measured by the effect of nalidixic acid (Lisle et al., 1999). A further method described by Holtmann and Sell (2001) for indirectly measuring the metabolic activity of biocide-treated bacteria is the determination of redox potential in the biofilm with microelectrodes. It can be shown that the increase of redox potential is correlated with a reduction in the colony count and the dehydrogenase activity. Microelectrodes are proposed as a way of monitoring the success of biocide treatments.

Less commonly, an additional measurement is used to determine the removal of biofilm bacteria. In this way, the surface-associated total cell count (i.e., active and inactive cells) can be determined microscopically following staining of the cells with fluorescent dyes. Suitable dyes for determination of total cell counts include 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), Acridine Orange (AO), Syto 9, and Sytox Green. These bind to DNA or, in some cases, also to RNA. Depending on biofilm thickness, the biofilm cells on a surface can be quantitated directly or after suspension and filtration onto a black (non-fluorescing) membrane filter. A further possibility for detection of surface-associated, stained bacteria consists in surface coverage by means of image analysis (Wirtanen and Mattila-Sandholm, 1996).

An assessment of the the biofilm thickness and internal structure can be performed by observation of the surface with a confocal laser scanning microscope (CLSM). With CLSM, any change in the morphology of the biofilm resulting from biocide treament can be detected. Often, an additional analysis of the surface-associated biofilm before and after biocide treatment is made using scanning electron microscopy (SEM).

5.1.5 Selected biocides

For information on the general activities and mechanisms of action of biocides, the reader is referred to recent reviews on this subject (McDonnell and Russell, 1999; Donlan and Costerlon, 2002) and to other chapters of this book. We have not attempted to present an exhaustive overview on the entire range of biocides and their efficacy against biofilms. Instead, some examples have been selected.

5.1.5.1 Chlorine-containing compounds [II, 21.2]

Because of its wide use in industrial water systems and clinical settings, much information exists about the effect of free available chlorine on biofilms grown in different environments. Other chlorine-based compounds which have been used in the control of biofilms are chlorine dioxide and chloramines. The term "chlorine" is broadly used to signify "active chlorine compounds" in aqueous solution, consisting of a mixture of Cl_2, OCl^-, HOCl and other active chlorine compounds (Dychdala, 1991). Chlorine is one of the most commonly used chemicals for the control of biofouling; however, side reactions with organic and inorganic compounds in the bulk water or with substratum material may result in substantial chlorine consumption and may render the biocide less effective; pH and temperature also have a strong influence on chlorine efficacy (Dychdala, 1991). Thus, water quality, choice of on equipment surfaces, but also hydrodynamic conditions are important factors that determine the efficacy of chlorine in water systems. An extensive process analysis of biofouling control with chlorine was given by Characklis (1990).

In water at pH values between 4 and 7, chlorine predominantly exists as hypochlorous acid (HOCl); it is in equilibrium with the hypochlorite ion (OCl^-), which predominates above pH 9. Hypochlorous acid is the rapidly acting component, and is mainly responsible for the microbicidal activity of chlorine. Thus it is suggested that low pH values enhance the biocidal activity, whereas high pH values (> 7) favour hypochlorite ion-mediated disruption and detachment of mature biofilms (Characklis, 1990). In this respect, data from the literature for chlorine efficacy on biofilms are difficult to compare because either different pH values were used or pH values are not mentioned. Moreover, most studies focus on the killing of biofilm organisms, while a limited number of studies also include the aspect of biofilm detachment from surfaces (Table 1).

In general, field observations and laboratory studies indicate that biofilms seem to be recalcitrant to the killing and disrupting activity of chlorine in concentrations which are relevant in practice, whereas planktonic cells are more easily inactivated by the biocide. Biofilm bacteria have been shown to persist in the presence of relatively high chlorine concentrations. For example, maintenance of a 1.0 mg/L free chlorine residual was insufficient to control coliform growth in drinking water distribution biofilms (LeChevallier et al., 1987). Experiments in a pilot plant showed that 4 mg/L free chlorine was not sufficient to kill bacteria in an established biofilm on iron surfaces (LeChevallier et al., 1990). After an exposure of biofilms on PVC pipe surfaces to free chlorine concentrations of 10 to 15 mg/L for seven days, biofilm bacteria (pseudomonads and mycobacteria) were shown by scanning electron microscopy (SEM) to survive in the form of EPS-enclosed microcolonies attached to the PVC walls (Vess et al., 1993); a few days after exposure to the disinfectant, the bacteria could be recovered again from the water phase.

Table 1 Efficacy of chlorine (sodium hypochlorite) against biofilms (examples). n.d., not determined

System	Material	Organisms	Age of biofilm	Product	Contact time	Concentration	Reduction in viable cell counts	Detachment of biofilms	References
Pipe system	PVC	pseudomonads, mycobacteria	8 w	sodium hypochlorite	7 d	10–15 ppm free chlorine	incomplete	incomplete	Vess et al., 1993
Tube system	silicone	tap water biofilm	50 d	chlorine	24 h	0.3 mg/L Cl_2, 10 mg/L Cl_2	none, 4 log units	none	Exner et al., 1987
Tube system	Tygon	*B. subtilis, E. coli, P. aeruginosa*	5 d	sodium hypochlorite	60 min	0.5% sodium hypochlorite	completely (>6 log units)	effectively (1% sodium hypochlorite)	Alasri et al., 1992
Batch system	stainless steel	*Pseudomonas* sp., *Listeria monocytogenes*	2 d	hypochlorite	1 min	160 mg/L	>8 log units	n.d.	Fatemi and Frank, 1999
Tube system	Tygon	*B. subtilis, B. megaterium*	>3d	sodium hypochlorite	5 min, 180 min	735 mg/L chlorine 735 mg/L chlorine 1439 mg/L chlorine	<1 log unit 2.5 – 2.8 log units	n.d.	Samrakandi et al., 1994
Tube system	PVC	*E. coli*	5 d	sodium hypochlorite	5 min	195 mg/L chlorine	>5 log units	n.d.	Ntsama-Essomba et al., 1997
Annular reactor	stainless steel	*P. aeruginosa, K. pneumoniae*	7–9 d	sodium hypochlorite	60 min	15 mg/L, pH 6.4 15 mg/L, pH 10.9.	41% reduction 99.6% reduction	47% removal 65% removal	Chen and Stewart, 2000
Flow system	stainless steel	*P. aeruginosa, K. pneumoniae*	6 d	sodium hypochlorite (pH 11)	60 min	1000 mg/L	0.85 log units	partially	Stewart et al., 2001

Exner et al. (1987) reported that 10 mg/L of free chlorine reduced the colony count of drinking water biofilms on silicone tube surfaces by approximately four log units after a contact time of 24 h; however, no complete inactivation was observed. SEM examination of the silicone surfaces demonstrated that no biofilm removal had occurred. A shorter contact time of 60 min or treatment with a lower chlorine concentration (0.3 mg/L) for 24 h had no inactivating effect on the biofilm bacteria.

Meiller et al. (2001) treated tubing sections from a dental unit waterline with bleach at the working concentration recommended by the manufacturer (0.52% sodium hypochlorite). After 18 h exposure, cultures were negative for biofilms, indicating antimicrobial efficacy of the product. However, SEM analysis revealed that the biocide failed to totally disrupt or remove the biofilms from the tube surfaces.

A minimum concentration of 0.5% sodium hypochlorite at a contact time of 60 min was necessary to achieve a complete inactivation of a mixed-culture biofilm (*E. coli*, *P. aeruginosa*, *B. subtlis*) on Tygon tube surfaces (Alasri et al., 1992); the same result was obtained with 2% sodium hypochlorite within 10 min. SEM analysis showed that application of 1% sodium hypochlorite for 60 min resulted in an effective detachment of the biofilms.

Chlorine has been reported to have limited efficacy against spore-containing biofilms (Samrakandi et al., 1994). Less than one log unit reduction of colony counts was observed when pure-culture biofilms containing spores of either *B. subtilis* or *B. megaterium* were treated with chlorine (735 mg/L, *B. subtilis*; *B. megaterium*, 1439 mg/L) for 5 min; even after a contact time of 180 min, colony counts decreased by less than 3 log units.

Flint et al. (1999) reported that for planktonic cells of *Streptococcus thermophilus*, no viable cells survived the exposure to 20 mg/L of sodium hypochlorite for 30 min (pH 6.8–7.0), whereas viability of this organism grown as a biofilm on stainless steel was still detected after treatment with up to 1 000 mg/L of sodium hypochlorite.

One-day-old biofilms of *P. aeruginosa* on silicone disks were inactivated to below the detection limit (> 5 log reduction of viable counts) after 10 min exposure to 0.1% sodium hypochlorite, or after 30 min exposure to 0.01% sodium hypochlorite, while the same degree of inactivation for planktonic bacteria was achieved in less than 1 min (Takeo et al., 1994).

Ntsama-Essomba et al. (1997) reported a minimal bactericidal concentration (decrease of colony counts by 5 log units) of 195 mg/L available chlorine when 5-day-old *E. coli* biofilms on PVC surfaces were treated for 5 min with a sodium hypochlorite-containing product; the minimal bactericidal concentration towards planktonic cells was five times lower. The age of biofilms influenced chlorine activity; the minimal bactericidal concentration towards 10-day-old biofilms was 10 times higher than for 5-day-old biofilms (Ntsama-Essomba et al., 1997). A similar observation was reported by LeChevallier et al. (1988a). Reduction of viable counts of 2-day-old *K. pneumoniae* biofilms was more than two log units higher than for 7-day-old biofilms, when the biofilms were exposed for 10 min with 1 mg/L of free chlorine (pH 7.0). No age-related increase in biofilm resistance was observed when the biofilms were treated with 5 mg/L of monochloramine under the same conditions (LeChevallier et al., 1988a).

Dual-species biofilms of *P. aeruginosa* and *K. aerogenes* grown for 6 days were highly resistant to killing by alkaline hypochlorite (Stewart et al. 2001). Treatment of biofilms with 1000 mg/L of the biocide for 1 h resulted in a 0.85 log reduction of the viable cell numbers, while similar treatment of planktonic cells led to a greater than 6 log reduction of the viable cell numbers. The average removal (65% of total cells) effected by hypochlorite dissolved in carbonate buffer (pH 11) was the same as the buffer without added hypochlorite. A similar observation was reported for biofilms grown in a continuous flow annular reactor system (Chen and Stewart, 2000). The efficacy of chlorine treatment at neutral pH was attributed to hypochlorous acid, while the efficacy of alkaline hypochlorite treatment was supposed to be due to the high pH value, and not the halogen (Chen and Stewart, 2000).

Using microelectrodes for monitoring biocide penetration into biofilms, a number of studies showed that transport of chlorine into biofilms was clearly retarded (de Beer et al., 1994b; Chen and Stewart, 1996; Xu et al., 1996; Stewart et al., 2001). This effect seems to be due to its interaction with cells and/or EPS in the biofilms, resulting in consumption and neutralization of chlorine. If chlorine has a higher reaction rate with biofilm components, then it will penetrate more slowly into the deeper layers of a biofilm. The chemical reaction between the extracellular polysaccharide alginate as one of the major EPS components in biofilms of *P. aeruginosa* and chlorine has been demonstrated (Wingender et al., 1999); *O*-acetyl groups in this alginate were mainly responsible for the reaction with chlorine, as removal of acetyl groups abolished the reactivity of the polysaccharide.

The chlorine-based biocides monochloramine and chlorosulfamates were reported to penetrate better into biofilms than free chlorine, because these compounds seem to have lower capacity for reaction with biofilm constituents (Griebe et al., 1994; LeChevallier et al., 1998b; Stewart et al., 2001). Although both compounds are weaker biocides than chlorine, they were shown to be similarly effective when tested against biofilms; however, their killing action was only very limited as was also observed for chlorine. The efficacy of chlorine and monochloramine was compared towards *P. aeruginosa* biofilms grown in an annular biofilm reactor (Griebe et al., 1994); a dose of 4 mg/L of monochloramine was found to be more effective for biofilm inactivation than a dose of 10.8 mg/L of free chlorine. Binary population biofilms of *P. aeruginosa* and *K. pneumoniae* grown for

7–9 days in a continuous flow annular reactor were killed more effectively by monochloramine than by free chlorine when treated with equivalent concentrations for 1 h at neutral pH (Chen and Stewart, 2000); however, the amount of biofilm removed by free chlorine and monochloramine was not statistically different. Rao et al. (1998) reported similar biocidal activity of monochloramine and free chlorine towards 2-day-old biofilms of seawater bacteria at similar doses of up to 3 mg/L and contact times of up to 60 min. The biofilm thickness was reduced by 90% in the case of both biocides; cell detachment was 94% and 100% for monochloramine and free chlorine, respectively (Rao et al., 1998). Yu et al. (1993) observed comparable disinfection efficiencies of 0.25 mg/L free chlorine (at pH 7.2) and 1 mg/L monochloramine (at pH 9.0) towards young *K. pneumoniae* biofilms; however, monochloramine was more effective in removing attached bacteria than free chlorine. LeChevallier et al. (1990) reported that biofilms in a model water distribution system were successfully controlled using monochloramine levels ranging from 2 to 4 mg/L; free chlorine residuals from 3 to 4 mg/L were ineffective in reducing the viability of biofilm bacteria grown on iron pipes. Based on these observations, it can be suggested that monochloramine is more effective against certain biofilms since it reacts more slowly in side reactions and thus is able to penetrate further into biofilms (LeChevallier et al., 1990).

Relatively few studies have included the effect of chlorine dioxide on biofilms. Characklis (1990) mentions that chlorine dioxide has been successfully used to control biofouling in several industrial environments. Walker and Morales (1997) studied the effect of chlorine dioxide on a mixed population of drinking water bacteria in a continuous culture model which was developed to simulate an industrial water system. The addition of 1 mg/L chlorine dioxide for approximately 18 h was sufficient to reduce the viable counts of the planktonic population by 99.9%, whereas 1.5 mg/L chlorine dioxide was required to achieve a similar reduction in the biofilms, suggesting an enhanced resistance of biofilm bacteria to the biocide. There are indications that continuous disinfection of drinking water using chlorine dioxide provides a certain control of biofilm formation. In a French drinking water distribution system, the presence of chlorine dioxide allowed a limited surface colonization, while in regions where chlorine dioxide was below the detection limit, an increase in biofilm formation occurred (Servais et al., 1995).

5.1.5.2 Ozone

Ozone has been used for the treatment of drinking water and wastewater, and has been proposed as a promising biocide in industrial cooling water systems due to its rapid action against planktonic microorganisms (Wickramanayake, 1991; Viera et al., 1999a). However, only few reports exist on the action of ozone against biofilms. In a study on *P. fluorescens*, the efficacy of ozone against sessile bacteria was lower than that found for planktonic cells (Viera et al., 1999a, b). Treatment of single-species biofilms of *P. fluorescens* with ozone (0.18–0.50 mg/L) for maximally 60 min resulted in a limited decrease in viable cell counts of only up to three orders of magnitude (Viera et al., 1999a); scanning electron microscopy revealed that the effect of ozone also included detachment of the cells of sessile bacteria from the steel surfaces. In the same study, viable counts in ozone-treated mixed-species biofilms of sulfate-reducing bacteria decreased by one to three orders of magnitude. The penetration of ozone into the *P. fluorescens* biofilms appeared to be a function of simultaneous diffusion in the biofilm matrix and reaction of the biocide with biofilm components such as cell mass, lysis products or EPS (Viera et al., 1999b). However, EPS may not always be reactive with ozone; thus, exopolysaccharides of *K. aerogenes* did not protect the bacteria from the action of ozone, since the sensitivity of encapsulated cells was not significantly different from that of an isogenic capsule deficient strain (Falla and Block, 1987).

5.1.5.3 Peroxygens

5.1.5.3.1 Hydrogen peroxide [II, 21.1.1.]. Hydrogen peroxide (H_2O_2) is being introduced more frequently into practice as a biocide due to its desirable environmental properties compared with the more conventional application of chlorine compounds. The breakdown products from the decomposition of hydrogen peroxide are water and oxygen, enabling simple waste disposal.

H_2O_2 has proven to be bactericidal against numerous Gram-negative and Gram-positive bacteria; against many viruses, hydrogen peroxide has a delayed action (overview in: Block, 1991b). In relatively high concentrations (10 to 25% w/v), hydrogen peroxide also displays sporicidal properties. It is assumed that the bactericidal activity of hydrogen peroxide functions principally through the production of hydroxyl radicals. The generation of hydroxyl radicals can take place through interaction with metal ions, e.g. iron (Fenton reaction) (Block, 1991). The highly-reactive hydroxyl radicals can attack membrane lipids, proteins, DNA, RNA and numerous other cellular macromolecules. Further susceptible positions are represented by sulfhydryl groups and double bonds in proteins and lipids, which are oxidized by hydrogen peroxide.

Hydrogen peroxide alone and in practice-relevant concentrations shows only weak inactivation efficacy against microorganisms in comparison with, e.g. chlorine; however, it does cause a dissolution and partial removal of biofilms (Exner et al., 1987; Christensen et al., 1990). It seems that intermittent application of

hydrogen peroxide leads to biofilms that are difficult to remove (Christensen et al., 1990). In dried surface assays with various bacterial species, hydrogen peroxide always demonstrates a good bacteriostatic effect against attached bacteria, but poor bactericidal activity (Baldry, 1983). For comparison, planktonic cells are also tested. The disinfection rate for planktonic cells is significantly higher compared with that of 20 h or older biofilms. The resistance of biofilm bacteria to hydrogen peroxide increases with the age of the biofilm (Cochran et al., 2000). An overview of the published work is shown in Table 2.

Other commercially available hydrogen peroxide based formulations also contain solubilised colloidal silver, whereby a synergistic effect is expected; an increase in the disinfection efficacy is claimed (Pedahzur, 1995). Various results have been published concerning the action of hydrogen peroxide and silver combinations. These mostly apply to laboratory investigations of suspended cells and pure cultures, where biofilms are not taken into consideration. It has been established that when silver ions are added to hydrogen peroxide, the combined action can be different from the individual components. In laboratory investigations, it has been shown that the combination of silver and hydrogen peroxide in a mass ratio of 1:1000 (in the range of 5 to 30 µg/L silver and 5 to 30 mg/L hydrogen peroxide) results in a higher inactivation performance in comparison to either component alone. Here, a weak synergistic effect has been described, such that the combined efficacy is higher than the sum of the individual inactivations through silver and hydrogen peroxide, respectively (Pedahzur et al., 1995). A similar synergistic effect has been observed in another investigation involving suspended cells (Potapchenko et al., 1996). A comparison of commercial hydrogen peroxide solution and a silver-containing hydrogen peroxide preparation (49.5% v/v hydrogen peroxide, 905 mg/L silver) suggests that there is no significant difference in the inactivation efficiencies against bacterial suspensions (Schiffmann, 1994).

5.1.5.3.2 Peracetic acid [II, 21.1.3.]. There are numerous studies concerning the action of peracetic acid which are again concerned with investigations of suspended bacteria (Flemming, 1984; Block, 1991b). Peracetic acid -containing products are commonly employed in the dairy and beverage industries due to their broad-spectrum microbial action and good ecological properties (e.g. degradability) (Orth, 1998). Peracetic acid is used in clinical environments for decontamination of surgical endoscopes and associated equipment as an alternative to the typically used glutaraldehyde (Ayliffe, 2000).

A series of publications deals with the action of peracetic acid on wastewater organisms (Mathieu et al., 1990; Sánchez-Ruiz et al., 1995; Rajala-Mustonen et al., 1997; Lazarowa et al., 1998; Liberti and Notarnicola, 1999). Here, the emphasis is on the potential practical applications of peracetic acid for disinfection, i.e. for inactivation of disease-causing organisms in the original sense. These results are also of interest from the point of view of action of peracetic acid on biofilms; it is assumed in this case that pathogenic organisms are present not as individual bacterial cells or virus particles, but rather as aggregates of faecal origin. Peracetic acid should also be active against disease-causing organisms in aggregated or immobilised form, similar to the case of biofilms.

The few publications which discuss the action of peracetic acid against drinking water or wastewater biofilms suggest that practice-relevant concentrations of peracetic acid cause a relatively fast and effective inactivation of biofilm bacteria, but result in no or minimal removal of the biofilm under these conditions. This is considered to be a disadvantage for at least the drinking water area, where residual biofilm material on surfaces can promote recontamination of the water (Exner et al., 1987; Morin, 2000). In this context, Mathieu et al. (1990) reported that the discontinuous application of peracetic acid led to an incomplete disinfection and dissolution of biofilms, but nevertheless can significantly limit the extent of biofilm development.

Alasri et al. (1992a) conclude that the combination of peracetic acid and hydrogen peroxide exerts a complementary effect on biofilms; peracetic acid has a clear microbicidal effect given that the hydrogen peroxide has a successful effect on biofilm detachment. The detached bacteria were significantly more sensitive to peracetic acid than the same bacteria in biofilms on the steel surface. It was therefore concluded that the complex structure of the biofilm and its attachment to the surface are responsible for the protective effect against peracetic acid, rather than a physiological adaptation of the biofilm bacteria with increasing resistance against peracetic acid (Johnston and Jones, 1995).

An overview of investigations concerning the effects of peracetic acid against biofilms is shown in Table 3.

5.1.5.4 Silver compounds

Silver and its compounds, including silver sulfadiazine and silver nitrate, have long been used as antimicrobial agents (Russell and Hugo, 1994). Some studies indicate that the incorporation of silver into paints and coatings for use in industrial water systems and in medical devices seems to be suitable to impair adhesion and delay biofilm formation, while in other studies no protective effect of silver has been observed.

Under the aspect of its application in water systems, a silver-containing paint was shown to retard surface colonization and growth of heterotrophic bacteria, including the pathogen *Legionella pneumophila*, for up to 14 days compared to control glass surfaces without the silver paint (Rogers et al., 1995). After prolonged incubation, the silver paint lost its protective effect against biofilm development, (Rogers et al., 1995) probably due to

Table 2 Efficacy of hydrogen peroxide (H_2O_2) against biofilms. n.d., not determined

System	Material	Organisms	Age of biofilm	Product	Max. contact time	Concentration	Killing	Detachment	References
Tube system	silicone	Tap water biofilm	50 d	H_2O_2	60 min	1.5 a. 2% H_2O_2	more than 5 log-units	partly	Exner et al., 1987
Tube system	Tygon-tube	*B. subtilis*, *E. coli*, *P. aeruginosa*	5 d	H_2O_2 (30/35% Produits chimiques du Ciron)	60 min	4% H_2O_2	completely (more than 6 log-units)	no	Alasri et al., 1992a
Hollow fibre	No information	*S. aureus*, *E. coli*, *P. aeruginosa*	1 h	H_2O_2 (30/35% Produits chimiques du Ciron) with PAA (Interox Chemicals)	4 d	97.65 mg/L H_2O_2 in combination with 0.75 mg/L PAA	completely (more than 6 log-units after 5 h contact time)	n.d.	Alasri et al., 1992b
Batch-reactor with pieces of pipes	stainless steel	Isolates from disinfectant dosage device	48 h	H_2O_2	60 min	1% H_2O_2	0.88 to 3.77 log units depending on the species	n.d.	Goroncy-Bermes a. Gerresheim, 1996
					3 h	1% H_2O_2	2.78 to > 5.82 log units depending on the species	n.d.	
Micro-annular reactor (MAR)	no information	*Pseudomonas* sp. NCMB 2021	30 h	H_2O_2	6 h	150 mM H_2O_2	n.d. (online monitoring with turbidity measurements)	84%	Christensen et al., 1990
		P. atlantica ATCC 19262		H_2O_2 and Fe^{2+}	6 h	1.5 mM H_2O_2 and 0.1 µM Fe^{2+}		72%	
Dried surface assay	steel	Spores of *B. subtilis* ATCC 15441	24 h (dried cells)	H_2O_2 (35% Interox Chemicals Ltd)	24 h	0.88 mol/L, pH 4–8	1 of the 14 tested carriers retains viable spores	n.d.	Baldry, 1983
Fed batch-reactor with alginate beads	alginate	*P. aeruginosa* PAO1	24 h	H_2O_2	1 h	600 mg/L H_2O_2	Disinfection coeff. 8.8 10^5 L/mg min	n.d.	Cochran et al., 2000
			48 h	1 h	600 mg/L H_2O_2	Disinfection coeff. 9.7 10^5 L/mg min			
Batch-reactor with cellulose carriers (3 M)	cellulose	*B. subtilis* (spores)		H_2O_2	20 min	11% H_2O_2	less than 6 log units	n.d.	Herruzo-Cabrera, 2000

Batch-reactor with coupons in poloxamer-gel	steel	*M. luteus*, *S. epidermidis*, *S. typhimurium*, *S. worthington*, *E. coli*, *L. monocytogenes*, *L. innocua*, *P. aeruginosa*, *P. fluorescens*, *B. subtilis*	5 h	H_2O_2/PAA	5 min	0.5% product	0.2 to 2 log-units	n.d.	Härkönen et al. 1999
Dried surface assay	Steel and silicone	*B. subtilis* subsp. *globigii* (spores)	dried cells	20% H_2O_2 and 4% PAA (Renalin)	11 h	1:5 dilution of the product	up to 5 log units	n.d.	Sagripanti and Bonifacino, 1999
Dried surface assay	Steel and silicone	*P. aeruginosa* ATCC 15442	dried cells	30% (Aldrich chemical Co.)	30 min	10%	2 log units (steel) and 4.4 log units (silicone)	n.d.	Sagripanti and Bonifacino, 1999
				20% H_2O_2 and 4% PAA (Renalin)		1:5 dilution of the product	4.5 log units (steel) and 6.4 log units (silicone)		
Batch-reactor (cassette) with 16 discs	Tylon and trylon with incorporation of Cu phthalo-cyanine (PC) or CoPC	*P. aeruginosa* PaWH	24 h	H_2O_2	30 min	3 mg/L H_2O_2	1.5 log units (control) 1.5 log units (CuPC) 2 log units (CoPC)	Biofilm on catalyst containing discs easier to remove	Wood et al. 1996

Table 3 Efficacy of peracetic acid (PAA) against biofilms. n.d., not determined

System	Material	Organisms	Age of biofilm	Product	Max. contact time	Concentration	Killing	Detachment	References
Tube system	Silicone	Tapwater-biofilm	50 d	Stabilized PAA, Tenside	60 min	0.5 u. 1% product	more than 6 to 7 log-units	no	Exner et al. 1987
Tube system	Tygon	Sewage-biofilm	4 d	PAA with H_2O_2	30 min 10 min	2.5 mg PAA/L 30 mg PAA/L	up to 97% n.d.	no ca. 40%	Mathieu et al. 1990
Activated carbon filter	Activated carbon	Mixed culture	45 d	PAA	10 min	100 mg PAA/L	not determined at the surface	partly	Morin, 2000
Tube system	Polyurethane-tubes	Tapwater-biofilm	14 d	PAA with H_2O_2 a. acetic acid (Spor-Klenz)	16 h	no information (solution ready for use)	100%	92.6%	Walker et al. 2001
Tube system	Tygon-tube	*B. subtilis*, *E. coli*, *P. aeruginosa*	5 d	PAA (Interox Chemicals)	60 min	0.5% PAA	completely (more than 6 log-units)	no	Alasri et al. 1992a
Batch-reactor with coupons	steel	*Pseudomonas sp.*, *Listeria monocytogenes*	2 d	PAA (Oxonia Active)	1 min	160 mg PAA/L	more than 8 log-units	n.d.	Fatemi a. Frank, 1999
Batch-reactor with coupons	steel	*P. aeruginosa*, *P. mirabilis*, *S. aureus*	4 h 20 h	PAA (Lever Industrial Ltd)	5 min 5 min	500 mg/L PAA 50 mg/L PAA 50 mg/L PAA	completely not completely (2-3 log-units) almost completely	n.d. n.d. n.b.	Johnston a. Jones, 1995
Batch-reactor with coupons	steel	*P. aeruginosa*	24 h	PAA (Proxitane, Solvay Interox R&D)	5 min	150 mg/L PAA	3 log-units	n.d.	Blanchard et al. 1998
Modified Robbins device	steel					30 mg/L PAA	3 log-units	n.d.	
Tube system	Tygon-tube	*B. subtilis*, *B. megaterium*	> 3 d	PAA (32%, Aldrich)	5 min 180 min	1222 mg/L PAA 1222 mg/L PAA	< 1 log-units max. 2.9 log-units	n.d. n.d.	Samrakandi et al. 1994
Tube system	PVC-tube	*E. coli*	5 d	PAA (2.5% with 18% H_2O_2, Bactipal)	5 min	125 mg/L PAA and 900 mg/L H_2O_2	more than 5 log-units	n.d.	Ntsama-Essomba et al. 1997
Batch-reactor with coupons	steel	*B. subtilis* *P. fluorescens*	34 h 24 h	PAA/H_2O_2	1 min 1 min	170 mg/L product 170 mg/L product	1 log-unit 2.5 log-units	n.d. n.d.	Lindsay a. von Holy, 1999
Batch-reactor with coupons in poloxamer-gel	steel	*M. luteus*, *S. epidermidis*, *S. typhimurium*, *S. worthington*, *E. coli*, *L. monocytogenes*, *L. innocua*, *P. aeruginosa*, *P. fluorescens*, *B. subtilis*	5 h	PAA/H_2O_2	5 min	0.5% product	0.2 to 2 log-units	n.d.	Härkönen et al. 1999
Batch-reactor with coupons	steel	*P. fragi*, *L. monocytogenes*, *B. thuringiensis*	6 d	PAA (Oxonia Aktiv) + alkaline cleaner	5 min	1% product	Decrease to approx. 10^3 cfu/cm^2	< 90%	Bredholt et al. 2001

Batch-reactor with papermill conditions	no information (reactor wall)	Isolate from a paper machine	48 h	PAA (5% with 23% H_2O_2, Proxitane 5, Solvay Interox)	point dosing	125 mg/L PAA	Increasing redox potential (indicates inactivation of several log-units)	n.d.	Holtmann a. Sell, 2001
Calgary biofilm device	polystyrol	*P. aeruginosa*	18 h	PAA	6 h	150 mg/L 300 mg/L	no killing more than 4 log-units	n.d.	Spoering a. Lewis, 2001
(Micretiter plates with pins)						400 mg/L	more than 6 log-units		

the resistance of silver from the surface of the paint. Silver-impregnated carbon filters are produced for point-of-use water treatment units. Based on literature data, it was concluded that, the average, no significant differences existed between silver-containing and nonsilver carbon units (Bell Jr., 1991). Silver-impregnated carbon units may become colonized with heterotrophic bacteria, which are released into the filter effluent, so that in these units silver does not usually confer a significant long-term effect on the treated water (Bell Jr., 1991). Silver coating has been suggested to prevent microbial growth on ion exchangers. Such coatings could suppress biofilm formation only for a period of a few weeks until a microbial population developed which could tolerate silver in much higher concentrations as actually present in the silver coated ion exchanger material (Flemming, 1987).

Adhesion and biofilm formation on catheters and implant materials by opportunistic microorganisms is a significant cause of infections patient in hospital. An approach to protect medical devices from microbial adhesion and biofilm formation has been to treat the surfaces with silver-containing coatings, to use silver-impregnated polymers or to produce silver ions electrically. The adhesion of Gram-positive and Gram-negative bacteria, and of the yeast *Candida albicans* was shown to be reduced on silver-coated catheters compared to non-coated controls (Gabriel et al., 1996; Gatter et al., 1998; Ahearn et al., 2000). Scanning electron microscopy (SEM) revealed that adhered cells of *P. aeruginosa* on hydrogel/silver-coated catheters after appeared damaged more often than on untreated catheters 18 hours (Gabriel et al., 1996). The biofilm development of *P. aeruginosa* monitored over 5 days was inhibited on catheters coated with silver at concentrations of 100 µg/mL and higher (Gu et al., 2001). In this study, the best protection against biofilm formation was achieved using a combination of 100 µg/mL silver and a lectin coating; this effect was probably due to the antimicrobial activity of silver ions and adhesion blockage from the lectins (Gu et al., 2001).

Electrically generated silver ions prevented the migration of *Staphylococcus epidermidis* through a silicone catheter over a 40-day period; this silver ion tophoretic catheter had a broad spectrum inhibitory activity against bacteria (*S. epidermidis*, *S. aureus*, *Acinetobacter baumanii*) and a yeast (*C. albicans*) (Raad et al., 1996).

Other authors reported no microbicidal effect of silver. The survival of three bacterial species and *C. albicans* on a silver-impregnated polymer was not found to be influenced by the silver incorporation (Kampf et al., 1998). Using confocal laser scanning microscopy, Cook et al. (2000) observed that a silver-coated prosthetic heart valve sewing cuff was colonized by a higher number of bacteria (*S. epidermidis*) than an uncoated cuff. McLean et al. (1993) reported that only a combination of silver and copper in multilayer surface films on catheter materials provided enhanced antimicrobial activity compared to uncoated or only silver-coated surfaces.

These observations show that depending on silver ion availability at surfaces of medical devices, adhesion and biofilm growth may be prevented or at least delayed, which may be beneficial, for example, for catheterized patients. A recently published meta-analysis of the effectiveness of silver-coated urinary tract catheters indicated a significant benefit in patients receiving silver-coated catheters; silver alloy catheters were significantly more effective in preventing urinary tract infections than were silver oxide catheters (Saint et al., 1998).

Silver and copper ions act synergistically in the killing of *Legionella* bacteria, which are known to multiply in biofilms in hot water distribution systems. Copper-silver ionization has been used successfully to control *Legionella* spp. in many US hospital hot water systems after 5 to 11 years of operation; however, high pH values and elevated chloride concentrations have negative effects on the biocidal efficacy of copper and silver, respectively, in water systems (Lin et al., 2002).

The effect of silver ion addition to established biofilms was investigated in several studies. Spratt et al. (2001) reported that treatment with 5 mg/L colloidal silver for 1 hour was ineffective in the killing of 48-hour membrane-grown single-species biofilms of root canal isolates. The colony counts in biofilms of *S. aureus* after exposure for 24 hours to silver sulfadiazine or to silver nitrate in rabbit plasma (at a silver concentration of 0.302%) were more than 3000 times lower than those in untreated biofilms (Akiyama et al., 1998). It was concluded that the silver compounds used had a bactericidal effect against immature (24-hour) biofilms. In a miniaturized biocide susceptibility test system, the bactericidal activity of silver nitrate was determined for biofilms of *Mycobacterium phlei* (Bardouniotis et al., 2001). The minimum concentration to kill biofilm organisms was 313 mg/L and 234 mg/L silver nitrate after exposure times of 30 min and 120 min, respectively. These observations suggest that exogenously applied silver ions can be effective against biofilms, but only at relatively high concentrations. Generally, no how long the effect lasted and how many times the treatment could be repeated with success.

5.1.5.5. Surface-bound biocides and "activated surfaces"

The approach to bind biocides to surfaces in order to prevent the development of biofilms seems to be very plausible at first glance, and some authors have invented and patented systems which either carry covalently bound biocides on surfaces or on which biocides are generated. Anti-fouling paints on ships fall into this category in that they rely on the controlled release of biocides from surfaces. An old example is copper plating in order to prevent fouling of ship hulls. The release of silver from surfaces belongs in a similar category, albeit not applied to ship hulls, and is discussed earlier. These examples demonstrate the principal problems of such approaches:

i. After a certain period of time, a natural selection process will yield strains which overcome the biocidal effect. In the case of copper, the first colonizers will be organisms tolerant to copper and may be later, in turn, colonized by copper-sensitive organisms.
ii. The biocide will be released into the environment and can result in harmful effects. An example is tributyltin anti-fouling compounds. However, these are so toxic to marine organisms that they have been widely banned from use.

5.1.5.5.1. Surface-bound biocides. Speier and Malek (1981) report that organosilicon-substituted organic amines, amine salts [II, 18.1.10.], or quaternary ammonium salts [II, 18.1.] displayed very effective antimicrobial activity when incorporated into solids. These authors did not claim that the biocide was insoluble but rather bound by specific adsorption to negatively charged groups on glass. They gave no information about how long the effect lasted or the capacity of the surface.

Hüttinger (1987, 1988) has presented a system in which biocides such as aldehydes, hydroxyaniline, aminophenylmercuryacetate, dibutyltinchloride and tributyltinchloride[II, 19.6.] were bound to cellulose as substratum, referring to earlier work of Isquith and McCollum (1978). The efficacy was evaluated by agar plate tests. From his results he concludes that only hydroxyaniline and tetrachlorobenzene, which were not active, had to be transported into the cytoplasm to be active. However, his study did not reveal whether this was only an effect of release of the other biocides into the medium which was less pronounced with the substances mentioned as ineffective. As plausible as this idea may appear in the first place, the approach must overcome a few serious problems:

1. If no biocide molecules are actually released, it is hard to understand how molecules which do not penetrate the cell wall can kill a microorganism. An explanation was not provided.
2. If cells attach a surface impregnated with biocides and are actually killed, there is not much probability that they leave the surface. Thus, the surface will be soon covered with dead bacteria and loose its efficacy.
3. Not only can bacteria adhere to the surface but also macromolecules of all kind. They may kinetically compete with adhering cells and mask the active groups, an effect which is called "fouling".
4. The authors claim a killing efficacy against planktonic cells also which seems to be a clear indication that the biocide must have been released into solution, as a remote biocidal effect of biocides covalently bound to surfaces conferred to suspended organisms is scientifically not understandable and it was not discussed by the authors.

Tiller et al. (2001, 2002) have covalently attached poly(4-vinyl-*N*-alkylpyridinium bromide) to glass slides and, thus, created a surface which supposedly kills airborne bacteria on contact. The authors do not provide evidence about the mechanism of action. It is quite probable that traces of the biocide are dissolved into the water droplets in which the test organisms have been sprayed onto the surface. Killing rates up to 99% are reported, which cannot, however, be considered as sufficient, as they include only two log steps.

5.1.5.5.2. Natural Anti-fouling Compounds. An interesting approach is to incorporate mechanisms with which living organisms defend themselves against colonization (Wahl, 1989). Natural anti-fouling compounds have been isolated mainly from some marine plants which are not colonized by bacteria (Terlezzi, 2000). Steinberg et al. (1997) have isolated signalling molecules antagonists (halogenated furanones) from an Australian seaweed which exhibits anti-colonizing activity. More marine antifouling products have been investigated by Armstrong et al. (2000). The problem with all of these compounds is that most of them are only scarcely available, that they are difficult to apply on a constant basis on a surface and that they will select for organisms which can overcome the effect. Apart from that, they will have to undergo the EU biocide guideline procedure which is assessed to cost currently about 5 million Euro per substance.

5.1.5.5.3. Surface-Generated Biocides. Another creative approach is the actual generation of biocides at a surface after which the biocide is released. Wood et al., (1996, 1997, 1998) observed that the activity of hydrogen peroxide or of potassium monopersulphate against attached bacteria was strongly enhanced if copper or cobalt phthalocyanine was incorporated into the surfaces, acting as catalysts for the formation of active oxygen species. The authors assume that the generation of these species at the substratum-biofilm interface concentrates the antimicrobial effect to the interfacial cells and generates a diffusion pump which further provides active species to the biofilm matrix. The survivors of low-concentration treatments with these agents were more readily removed from the catalyst-containing disks than from control disks. This indicated advantages gained in hygienic cleansing of such modified surfaces. This approach was expanded to thick biofilms (100 μm) of *P. aeruginosa* and still yielded significant enhancement of killing which originates from the interface. Reaction-diffusion limitation seems to concentrate the active species within the biofilm rather than protecting it, which could explain the efficacy against thick biofilms. A further advantage may be that EPS molecules can be disrupted, thus weakening the adhesion to the substratum and leading to biofilm detachment.

5.1.6. Conclusions

The "real world" which biocides encounter when employed will be biofilms, not suspended pure cultures, because this is the preferred mode of life of microorganisms. In biofilms, they have developed many ways to increase their resistance, as discussed earlier. From an ecological view, this makes perfect sense as the organisms have had to defend themselves against adverse conditions since the evolution of life. The protection mechanisms evolved during this time had to deal with all kinds of biocidal effects as occurring under natural conditions. Therefore it is not surprising that man-made biocides usually work better in the laboratory than under practical conditions. The possibility of enhanced resistance of biofilm organisms thus always has to be considered.

It is impossible to provide detailed and predictable protocols for disinfection of biofilms in various situations which can be generalized. In principle, biocides can be effective against biofilms. However, this has to be verified in given situations and it has to be taken into consideration that the three crucial factors for biocide efficacy, i.e., concentration, exposure time and temperature, have to be optimised in order to achieve the required efficacy. It will be important to verify the effect. In order to do so, it is necessary to perform the assessment with intact biofilms and not with removed and suspended biofilm organisms as these will be much more susceptible to the biocide.

Another important aspect is to distinguish between disinfection and cleaning. Biocides are designed for killing microorganisms. However, killing the organisms and leaving the biomass in place will in most cases not solve the problem but rather lead to rapid aftergrowth. The fact that some of them actually disrupt biofilms and contribute to their removal cannot be extrapolated to all biocides.

These considerations may explain why the application of biocides in technical systems is sometimes not successful. In order to achieve success, integrated approaches (Cloete and Broezel, 2002; Flemming, 2002) are recommended. These include:

i. Detection of biofilms on surfaces
ii. Application of biocides and cleaners
iii. Verification of killing efficacy and removal of biofilms

Interestingly, practically all studies on disinfection of biofilms focus on already existing biofilms and not on their prevention. Biocides can be effective in prevention of biofilm formation, as practical experience with chlorine in swimming pools proves. This aspect has not been well covered scientifically.

For optimizing the use of biocides against biofilms, it will be useful to monitor their development on surfaces *in situ*, on line, in real time and possibly in a way which can be automated. Some devices have been developed which fulfil these requirements (Flemming, 2003; Tamachkiarow and Flemming, 2003).

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Exhibit C

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An automated system for biocide testing on biofilms*

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The paper presents and discusses a novel on-line real-time non-destructive continuous-flow system for biocide testing on industrial biofilms. This laboratory system is capable of monitoring changes in growth, accumulation and respiratory activity of biofilms in response to biocidal treatment. The system incorporates a fouling monitor for continuous measuring of the rate of biofilm accumulation (heat transfer resistance), a sensor for monitoring of microbial activity (oxygen meter for monitoring the rate of biofilm respiratory activity), and subsystems necessary for microbial life support and control of operation parameters. Examples of system operation and testing of oxidizing and non-oxidizing biocides are presented.

Keywords: biocide; biofilms; *Sphaerotilus natans*; heat transfer resistance; dissolved oxygen

Introduction

Control of biofilms in cooling water systems is an important component of any successful industrial water treatment program. Although most microorganisms in industrial systems are associated with surfaces, biofilms have historically received less attention than planktonic microorganisms. However, it has been shown that various biocides are less effective against sessile microorganisms than against free-floating dispersed cells [1,11,12]. The resistance of biofilms to antimicrobials, combined with their complex architecture and dynamic nature, make biofilms quite difficult to measure, monitor and control, and thus reduce the effectiveness of treatment strategies. Therefore, monitoring and control of biofilm accumulation is a challenging task to industry.

There are many known procedures for the determination of antimicrobial effectiveness on biofilms [4,8,9,21]. Success in selecting test methods depends on understanding which criteria are most important in a particular system, the ability to develop reproducible biofilm, and simulating applicable field conditions. The recent tendency in biofilm research is to go 'high-tech' using sophisticated techniques in order to understand structure, functions and response of biofilms on the microlevel, including confocal laser microscopy, spectrochemical, electrochemical and piezoelectric techniques [8,17]. However, in industrial applications, accurate information on the relative efficacy of biocides on biofilms is sufficient in most cases.

It is recognized in the water treatment industry that the most reliable data could be obtained via continuous, on-line fouling monitoring techniques. Generally, there are two types of continuous-flow systems currently used for the evaluation of biocidal efficacy on biofilms. Systems of the first type are usually a combination of a chemostat and a series of removable coupons (usually Robbins device, modified Robbins device, rectangular or tubular coupons) under continuous-flow and nutrient addition conditions. These systems utilize destructive techniques based on dry weight, plate counts, DNA, ATP, INT or other methods as a measure of accumulation or microbial activity [2,10,15,16,20]. Another system type is a continuous, on-line fouling monitoring technique [1,19,23]. Such a system is typically restricted to measuring the effects of the fouling deposit, with no evaluation of the biological components or direct measurement of biological accumulation. The mission of such a system is usually as field support of a biocide treatment program or as a process control program for mitigating the effects of biofouling/biocorrosion. The present use of fouling monitors in industrial applications focuses on methods incorporating changes in heat transfer resistance, differential pressure, fluid frictional resistance. Optical, acoustic, and other methods for indicating accumulation are in development, or are used in rare cases [1].

In general, there is a lack of information on the use of on-line biofilm monitoring techniques for biocide evaluations in defined laboratory conditions. Meanwhile, such an approach could be useful in understanding the mechanisms of biofilm accumulation, removal and inhibition, and could lead to the development of new biocides.

This paper presents a laboratory system which uses established industrial monitoring techniques for accurate monitoring of biocide impact on biofilms. The system incorporates a fouling monitor for continuous measuring of the rate of biofilm accumulation (heat transfer resistance), a sensor for monitoring of microbial activity (oxygen meter for monitoring of the rate of biofilm respiratory activity), and subsystems necessary for microbial life support and control of operation parameters.

Method development

Selection of components for system design

Analysis of available technical literature [1,5,19,23] showed that no one ideal monitor exists that will provide complete information on fouling accumulation, effects, and treatment efficacy. The approach chosen was to integrate specific established industrial on-line monitoring methods with specially developed operational procedures for biofilm

*The computer simulation of this system was presented at ASM Meeting on Microbial Biofilms in Snowbird, UT, in October of 1996

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development and testing, into a specialized laboratory system for biocide evaluation.

The Heat Transfer Resistance (HTR) method of biofilm monitoring was selected as a prime monitoring technique. This method relies on detecting the increased HTR associated with the deposit. Various configurations are described in the literature [6,18,22], but all are based on inducing a heat load on a test section and monitoring the temperature change over this test section at a given flow rate. Temperature in the test section increases as a fouling deposit thermally insulates the surface and restricts transfer of the heat load. This system requires constant flow and induced heat load which is relevant to a real-life system if the system of interest includes a heat exchanger. The interpretation of HTR data is based on the fact that the overall heat transfer resistance consists of two components: conductive HTR and advective HTR. Conductive HTR is dependent on the thermal conductivity and generally increases as the biofilm accumulates. Advective HTR results from fluid motion or turbulence and generally decreases as biofilm accumulates, since the roughness of the biofilm increases turbulence in the interfacial region. Many results obtained with DATS Fouling Monitor in industry suggest that the assumption of correlation of biofilm accumulation with conductive and overall HTR appears valid [25]. The second method incorporated in the biofilm monitoring system was continuous measurement of dissolved oxygen (DO) in the water surrounding the biofilm that correlates with the metabolic activity of aerobic bacteria. Measurement of respiration rates of microorganisms was used previously for determination of relative effectiveness of biocidal activity of different biocides [3]. The concept behind the developed system for biocide testing was based on the fact that the system's make-up water was kept at constant oxygen saturation level (by continuous sparging of air) and constant pH and temperature. Thus, any changes in the dissolved oxygen concentration of the recirculating water were considered to occur due to biofilm activity. As an additional measure of biofilm activity, the measurement of pH of the circulating water was performed. It was anticipated that the change in pH would follow the DO curve because the CO_2 released during respiration would increase water acidity, and accordingly, would reduce the water pH.

System setup

A continuous-flow heat-exchange loop (HEL) was developed to establish and support biofilm growth (Figure 1). To produce an environment conducive to the production of a voluminous heavy biofilm, a biological growth reactor or chemostat was used. The reactor was a 5-gallon tank (11.0 liters working volume) equipped with stirrer, drilled and fitted with PVC piping and joints for inflow and overflow connections. The setup included three subsystems: (a) microbial life support; (b) measurement; and (c) control.

Microbial life support subsystem: The microbial life support subsystem was connected to the chemostat by nutrient feed (nutrient vessel, tygon tubing, pump, flow-breaker and valves), make-up feed (water tank, tygon tubing, pump, immersion heater, air sparging, floating valve), and chemical feed (reservoir, pump, tygon tubing, valves). The chemostat was also connected to a circulation pump that circulates liquid through the 316 stainless steel metal tube (61 cm in length and 1.59 cm in diameter) incorporated in a heat exchanger, which is a part of the DATS Fouling Monitor and simulates the actual heat loading present in industrial heat exchangers. In order to dissipate the heat from the heat exchanger, an additional chilled water cooling system, consisting of a cooling coil connected to a refrigerated circulating bath, was introduced (Lauda RC20, Brinkmann Instruments, Westbury, NY, USA).

Measurement subsystem: The major elements of this subsystem are the DATS II Fouling Monitor from Bridger Scientific (Sandwich, MA, USA) [7], and a TBI-Bailey (Carson City, NV, USA) TB 701 analytical controlling transmitter coupled with a dissolved oxygen probe TB234 and pH probe TB 551. The sensor elements, ie oxygen sensor and pH-probe, were introduced into the chemostat. Temperature probes located in the heat exchanger area measured the water and heater block temperatures, while a sensitive flow meter measured the water flow velocity through the steel tube. The HTR was automatically calculated from these measurements and heat load. Through an analytical controlling transmitter, 4–20 mA signals from the DO and pH probes were sent to the auxiliary input channels in the Data Acquisition System. Thus, DO and pH levels were easily monitored and logged along with temperatures and other fouling monitor data. Data were collected continuously by a personal computer. A customized LabVIEW based software interface simulated the configuration of the operating conditions, collected, stored and displayed the data. Variables were scanned every 15 s, and the average recorded every 3–60 min in a data file, which was automatically transferred to Excel for analysis and graphing.

Control subsystem: There were several levels of controls in the present design of the system. The DATS controller provided control of the flow rate, heat load or wall temperature. Through the water chiller (RC20), stability of the recirculation water temperature was achieved. Overflow tubing in the chemostat secured the water working volume. The presence of a floating valve, in combination with continuous sparging and temperature control in the make-up reservoir made it possible to supply the system with aerated water of constant temperature.

The program was formed in such a way that the system functioned continuously without interruptions for several weeks. Only biofilm growth in the tank, with possible clogging of the outlet tubing, could cause unforeseen maintenance problems. Regular maintenance, including nutrient preparation, make-up and nutrient flow rates check-up and calibration, check-up of pumps, tygon tubing conditions, etc, should be provided on a daily/weekly basis.

Growth conditions

The sheathed *Sphaerotilus natans* (ATCC 15291), which is known to reside on heat exchanger surfaces in cooling water systems and papermaking machines, and is also associated with sewage treatment process upsets (such as the bulking of activated sludge), was selected for biofilm growth. This organism was successfully grown as a biofilm in several studies [16,24]. Details of inocula preparation

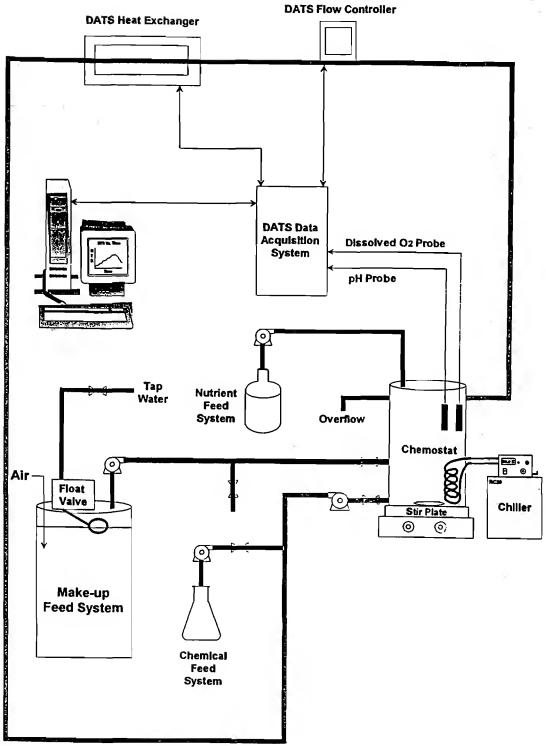

Figure 1 An automated system for biofilm monitoring.

and growth conditions in the static system were described earlier [13]. Prepared inocula were pumped into the microbial growth reactor and usually allowed to sit at room temperature overnight. The next day the make-up water (Clinton tap) and nutrient (CGY media) were started. The selection of initial growth conditions and system parameters was based on previous experience, laboratory limitations, geometric size of the system components, and the desire to promote a growth of biofilm. Shifting of growth conditions from planktonic growth to attached filamentous growth was obtained by lowering media concentrations to 5% or less and maintaining dilution rates higher than the maximum specific rate (according to [24]). Selected test conditions are shown in Table 1.

Table 1 *Sphaerotilus natans* biofilm growth conditions

Inoculum	*S. natans*
pH	7.2–8.2
Circ. water temperature	75°F
Wall temperature	85°F
Make-up water	Clinton tap 150–200 ml min^{-1}
Substrate	CGY media 0.5–1.0 ml min^{-1}
Water flow	1–5 f.p.s.
Half-life	45 min

Monitoring of biocide efficacy

Monitoring of biocide efficacy was performed on already established biofilm. The treatment procedure was designed in such a manner that the first treatment would be performed at the point where the slope of HTR rises to the level of $2.0–2.5 \times 10^{-3}$ h-sq ft-F btu^{-1}. Two biocides were tested in the biofilm monitoring system: non-oxidizing and oxidizing biocide. During these runs the system function was tested at constant conditions. The wall temperature was kept at 85°F, the flow rate at 3 fps, and nutrients were fed at 0.5–1.0 ml min^{-1} level. Make-up and discharge rates were kept at 170 ml min^{-1}, and the dilution was close to 0.9. Biocide treatment was carried out by: an initial slug dose injected to overcome biocide demand, followed by a continuous 3-h maintenance treatment in concentrations, calculated per make-up water. Such treatment was repeated for 3 consecutive days, every 24 h.

Results and discussion

Biofilm growth

There are many parameters that are important for successful biofilm growth. Parameters such as water/surface temperature, flow rate, and nutrient level were of great importance in the current design. The results of testing and optimization of these parameters are discussed further.

Effect of temperature: The first series of experiments was designed to define the system parameter stability, limitations and operation range in 'no-growth' and 'growth' conditions in the designed configuration. The first task was to define temperatures for optimal biofilm growth. It was known that the optimal temperature for *S. natans* is 75–85°F and that the DATS fouling monitor can operate in two different modes: (a) constant heat load (usually used mode); and (b) constant wall temperature (the heat load is automatically adjusted by the DATS controller to compensate for the insulating effect of the biofilm). From 'no growth' tests it was found that the bulk water temperature changed much more slowly than the block and wall temperatures with increasing heat load, and that the working volume and flow rate had a less significant effect on the temperature parameters than the heat load. It was demonstrated that due to the insulating effect of grown biofilm, the wall temperature but not water temperature was the limiting parameter in biofilm growth at the designed configuration. The conclusion was made to continue biofilm growth experimentation under constant wall temperature conditions.

Effect of flow rate: The test (Figure 2) involved varying the flow rate (from 1 to 5 fps) to determine its impact on biofilm growth and to optimize conditions for biofilm growth. The test demonstrated that the HTR was responding adequately to changes in flow velocity. In general, when the flow rate was increased, sloughing occurred. The sloughing event was also confirmed by the increase in the bulk water turbidity corresponding to HTR decline. The significant regrowth was observed several hours after each sloughing. It was found that the higher flow velocity caused more significant sloughing. Additional experiments demonstrated that the optimal biofilm growth could be obtained at a flow rate of 3 fps which is typical for heat exchangers in many industrial applications. This flow rate was selected for further experiments.

Typical biofilm growth: Figure 3 shows typical patterns of HTR, DO and pH changes corresponding to attached growth of *S. natans*. The HTR curve showed significant exponential growth, while the DO level reduced sharply, according to strong respiration of the biofilm organisms in the presence of nutrients. The pH curve followed the DO curve. Nutrient addition and stop events had a very strong effect on biofilm growth. There was no growth in the system without appropriate nutrient addition. On the other hand, an excessive nutrient addition supported undesired planktonic growth and increased halogen demand of the bulk water which was a negative factor. Nutrient addition was optimized at 0.5–1.0 ml min^{-1} of CGY medium. The stop of nutrient addition was followed by a reduction in respiration rate. Reduced respiration initiated an increase in aqueous dissolved oxygen concentration due to saturation of the make-up water with oxygen. The theoretical exponential curve, calculated from an assumption that there is no biofilm in the system, and based on the half-life calculations, shows that about 5 h would be required for the system to go from zero to almost 100% DO, as well as to achieve the make-up water pH 8.2. These parameters would stay at that level without biofilm. In the presence of biofilm, it took about 20 h to reach a steady dissolved oxygen level of about 85%, probably corresponding to endogenous respiration only. As soon as the nutrient pump was turned on again, DO and pH levels sharply decreased to the level they had before the nutrient was stopped. Somewhat different changes happened to HTR level: a sharp decrease in the first several hours was followed by a slow steady decrease which continued until the nutrient was turned on. At this point, HTR began to increase, but at a pace slower than during initial growth phase in the first 72 h. Biofilm probably 'takes time' to recover from nutrient starvation. Evidently, an erosion of biofilm takes place without nutrient, followed by biofilm regrowth when nutrient is restored.

Effect of biocide: Figure 4 demonstrates the typical effect of an effective biocide on biofilm. In general, the behavior of the curve is similar to the nutrient stop: there are three portions on most curves. The first part usually

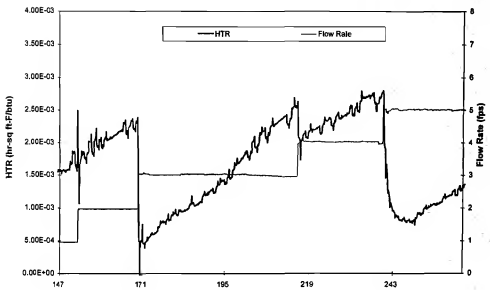

Figure 2 Effect of flow rate on the growth and sloughing of *Sphaerotilus natans* biofilm. Sharp declines in HTR values correspond to biofilm sloughing events.

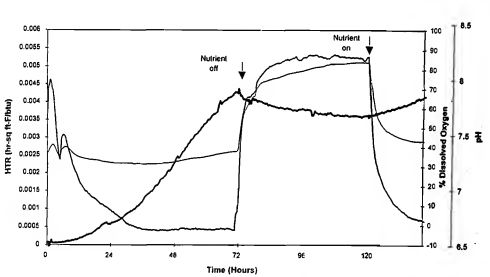

Figure 3 Typical biofilm growth of *Sphaerotilus natans* biofilm under described conditions. Arrows correspond to the start and stop of nutrient addition.

shows the decrease in HTR level and increase of DO and pH, probably corresponding to erosion or removal of biofilm and a cessation or reduction in respiration. The second part of the curve could show a plateau in parameter value, corresponding to suppression of biofilm growth (some level of kill). The third part shows an increase in HTR, DO, and pH levels, corresponding to biofilm regrowth. It was anticipated, that depending upon the nature of the biocide, the mechanism of its activity and concentration, the shape of HTR, DO and pH curves would indicate the biofilm response to the biocide. By comparing these curves to curves obtained previously, a definition of biocidal efficacy can be obtained. In general, HTR data are produced only by biofilm grown on the heated portion of the tube (15.2 cm in length), while biofilm covers most of the system surface, and DO and pH data are the response from the whole system.

Non-oxidizing biocide: Isothiazolone at 4 ppm active was used in this test. It was known that, by mechanism of action, this biocide influences respiration. Figure 5 shows that the first treatment with this biocide was effective in reducing HTR (removing some biofilm), and increasing the DO level to about 70% of the level of saturation. However, within 24 h, evident regrowth occurred, and the second treatment was much less effective than the first. The effect from the third treatment was less effective than the second. From this and other experiments an observation was made that the biocidal efficacy of non-oxidizing biocides diminishes with every subsequent treatment. The reasons for this

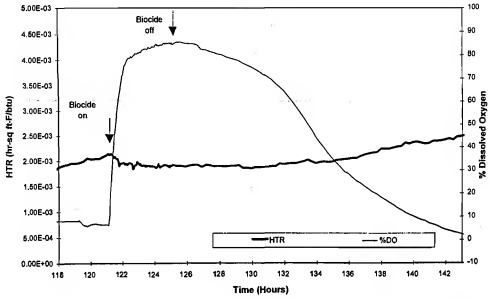

Figure 4 Effect of biocide treatment on *Sphaerotilus natans* biofilm. Arrows correspond to the start and stop of biocide addition.

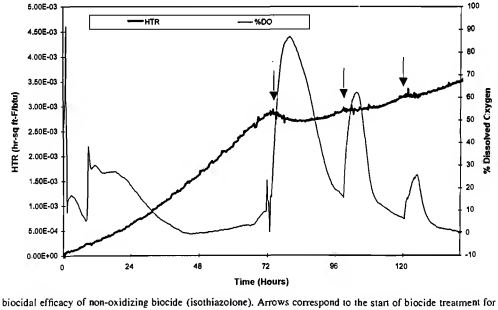

Figure 5 Testing biocidal efficacy of non-oxidizing biocide (isothiazolone). Arrows correspond to the start of biocide treatment for 3 consecutive days.

fact are not clear. There are several possible explanations: (1) an increase in biomass affects biofilm response; (2) physiological acclimation/adaptation of the biofilm cells to biocides; (3) a reduction in the biocide transport permeating the biofilm due to extensive growth of biofilm; (4) an improving transport of nutrients to living cells following treatment with biocides, etc.

Oxidizing biocide: A slow-release oxidizing biocide containing methylethylhydantoin, bromine and chlorine was dosed at initial concentrations of 10 ppm as total Cl_2. Results of the biocidal treatmens are shown in Figure 6. In the case with oxidizing biocide, suppression of biofilm accumulation was followed by fast regrowth. On the other hand, reduction in respiration response of biofilm to the second and third treatments was less expressed than in the experiment with non-oxidizing biocides. Probably, these experimental results reflect the difference in biocidal mechanisms between non-oxidizing and oxidizing biocides. Additional data on the comparative performance of oxidizing biocides can be found in [14]. On-going efforts are directed towards understanding and interpreting results, as well as towards testing of new biocides and their blends.

Conclusions

(1) The design and monitoring methodology of biofilm monitoring system proved to be successful.
(2) On-line measurements of heat transfer resistance, dis-

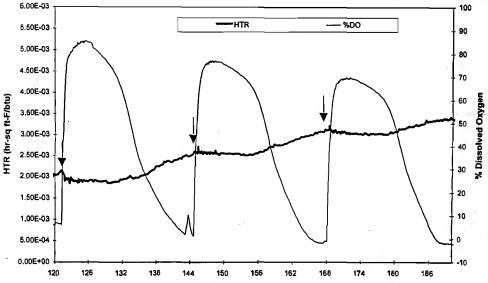

Figure 6 Testing biocidal efficacy of oxidizing biocide (halohydantoin). Arrows correspond to the start of biocide treatment for 3 consecutive days.

solved oxygen, and pH data provided quantitative information on biofilm accumulation, removal, and biofilm microbial activity.

(3) This technique demonstrated the capability to detect and record, in real time, the impact of the biocide treatment on biofilm growth.

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For: METHOD FOR REMOVAL OF BIOFILM | Examiner: P. A. Hruskoci

DECLARATION OF MICHAEL LUDENSKY, PH.D. UNDER 37 C.F.R. § 1.132

Exhibit D

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Control and monitoring of biofilms in industrial applications

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1. Introduction

Control of biofilms in industrial water systems is an important component of any successful water treatment program. Although most microorganisms in industrial systems are associated with surfaces, biofilms have historically received less attention than planktonic microorganisms. However, it has been shown that various biocides are less effective against sessile microorganisms than against free-floating dispersed cells. The tolerance of biofilms to antimicrobials, combined with their complex architecture and dynamic nature, make biofilms quite difficult to measure, monitor and control, and thus reduces the effectiveness of treatment strategies. Therefore, monitoring and control of biofilm accumulation is a challenging task to industry.

Because of the complexity and uniqueness of each water-based technological process and a lack of clear understanding of biofilm behavior, successful long-term biofilm control programs often involve several biocides and specific cleaning/sanitation regimes and seem to be more an art than a science. Neutralization of biocides by biofilm is almost inevitable problem of any control program. In practice it means that fast biofilm regrowth and proliferation follow any inadequate control treatment regime.

The objective of this paper is to demonstrate biofilm-related problems in three different applications, discuss response of laboratory-grown model biofilms to oxidizing and non-oxidizing biocides treatment, and recommend specific biofilm control modes with currently available biocides for discussed applications.

2. Biofilms in industrial applications

In spite of the fact of tremendous developments in biofilm research in recent years, industry has not completely embraced biofilms as a source of microbiological problems. It is rather "fouling" or "biofouling" that is industrial word for biofilms. On the contrary, industrial biologists who deal with fouling issues in the field blame on biofilms most of microbiological problems in the industrial processes. In any case, it is becoming increasingly clear that biofilms rather than planktonic microorganisms cause essential damage to water based technological processes. A review of biofilm monitoring techniques for industrial applications has been published earlier (Ludensky, 1999). However, practical monitoring and evaluation procedures for testing efficacy of biofouling control in the field are still based on planktonic evaluation.

It is almost impossible to talk about biofilm-related problems in industry in general. The properties of biofilm vary with specifics of technological process where biofilms grow/occur, as well as with environmental factors such as surface materials, nutrient conditions, hydrodynamics, source of microbiological contamination, species distribution, etc. (Characklis and Marshall, 1990). From practical point of view biofilm is a complex dynamic organic polymer structure, which is developed and constantly changed by community of living in it microorganisms. There are at least three features (properties) of the biofilm that effect the environment and normal functioning of industrial technological process: physical (structural), chemical (metabolic) and biological (living). Each of these biofilm elements can effect industrial technological process. For example, biofilm structure of bacterial extracellular polymers may cause an unusually high fluid frictional resistance in water conduits, metabolic activity within biofilm may change chemistry of the environment and initiate microbiologically induced corrosion or odor problems. On other hand, being a consortium of living microorganisms, biofilm serves as a depot of potential contamination problems or resurgent infections. We will discuss at a greater length, biofilm-related problems in: (1) industrial cooling water system, (2) industrial process water (white water) in papermaking, and (3) manufacturing of consumer/cosmetics products.

2.1. Cooling water systems

A recirculating cooling tower system is a necessary part of any industrial process. In such a system microbial growth could be very high due to the presence of nutrients, favorable temperatures, high residence time, high ratio of surface area to the volume, etc. In general a cooling tower is an ideal

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place for the growth of living organisms, because it provides air, heat and light. Many forms of living organisms proliferate here (Cloete et al., 1992). The major economic impact caused by biofilms in cooling water systems is because of energy losses due to increased fluid frictional resistance and increased heat transfer resistance at power plant condensers and process heat exchangers. Biofilms accumulating on the surfaces of heat exchange tubes significantly reduce the heat transfer rate because the thermal conductivity of biofilms is significantly less than that of metal heat transfer surface materials. Other negative impacts of fouling include increased capital costs for excess equipment capacity and premature replacement of equipment experiencing MIC, unscheduled turnarounds or downtime to clean equipment that fouled, as well as safety problems, including Legionella related problems.

The objective of biocide treatment at cooling water system is usually to keep heat exchangers relatively clean from any biofouling (biofilms). Usually, this is achieved by continuous or periodic slug dosage of oxidizing biocides. Monitoring parameter is usually number of CFU per ml of cooling water. If this number is 10^4 and below, the control program is considered to be successful.

2.2. Paper manufacturing

Paper manufacturing is a water-based process, employing a range of raw materials that introduce a vast array of microorganisms into a warm, nutritious environment. The growth of such microorganisms on the surfaces of process equipments is often as a thick biofilm or "slime". The development of slime is especially common within a paper machine, particularly at interfaces between the machine and water. Microbial growth and slime formation in the paper mill environment produces a wide range of operational problems for the paper maker: (1) wet end breaks causing runnability problems; (2) sheet defects such as spots, holes and blotches from slime sloughing onto the circulating wire; (3) odors both in the mill and carried over onto the finished paper, etc. (Elsmore et al., 1999). It is estimated that 10–20% of all machine downtime is caused by slime problems. There can be no consensus on the microbiological content of paper mill whitewater, since the sources of contaminants are many and varied. The main source of microorganisms in the process is the water used. In enclosed systems microorganisms will enter the process water from its initial source, and will find an ideal environment for growth and reproduction, with the increase of nutrients and metabolites. Slime formed within the plant then becomes the major source of microbial challenge. Another significant source of bacteria is recycled pulp. Microorganisms will also be associated with additives, fillers, dyes, pigments, starches and coatings, which can also differ with the types of paper produced and between mills but which will each bring their own characteristic flora. The reported flora associated with paper processing includes mostly aerobic and anaerobic bacteria. Total aerobic counts typically range between 10^4 cfu/ml for high grade paper under tight biocide control, through to 10^8 cfu/ml for poorly controlled lower quality papers such as brown paper and cardboard. Collected reports on the nature and microbiology of the slime (Elsmore et al., 1999; Blanco et al., 1996) report a markedly different range of species to that of white water with *Bacillus sp.*, and *Sphaerotilus natans* predominating. For most paper mills if the bacterial content in the white water is less than 10^6 cfu/ml, it is considered that slime is under control. Methods of control vary between mills, however, most of mills use non-oxidizing biocides as slime control agents.

2.3. Manufacturing of household/cosmetics consumer products

On the contrary to the cooling water systems and papermaking, which are mostly continuous processes with a relatively high level of water closure, manufacturing of household/cosmetics consumer products is based on a batch processes without any recirculation of water. Manufacturing of household /cosmetics consumer products is essentially mixing of several chemical ingredients with variable water content, storage and packaging in the end-use product. The major problem for this products is potential contamination with water-borne bacteria and fungi which is often based on development of resistance to commonly used preservatives, that are added to the end-use product. It was demonstrated that microorganisms that localize in biofilms in different locations within the manufacturing plant and are usually associated with pipelines, dead legs, and water storage tanks cause microbiological contamination. The pipe work in a plant is considered to be a major source of problems. It is not possible to open it up and have a look inside, and the amount of contamination from pipe work is often significant. Once contaminated, pipe work is the hardest part of a plant to get clean and free from infection. There are practically no references in available technical literature on biofilms in manufacturing processes. The major emphasis in biofilm control in this application is on avoiding contamination through maintaining cleanliness of the plant and on plant hygiene. It is recommended to continually monitor the hygiene status of the plant, including visual inspection, and periodic sampling for bacterial counts. The specific acceptable levels of cfu/ml vary between companies and plants but 10^3 cfu/ml is considered to be a general number corresponding to relative cleanliness of the plant.

3. Experimental

3.1. System setup

An on-line testing system for biocide efficacy testing was used to provide a real-time, non-destructive method for biofilm monitoring and measurement of biocidal

efficacy. The system design was based on monitoring the indirect parameters which correlate to biofilm formation and accumulation, such as heat transfer resistance (HTR), and to changes in biofilm activity, such as dissolved oxygen (DO) in the bulk water. The system consisted of a continuous-flow heat-exchange loop, a biological growth reactor and subsystems for life support, biofilm measurement, and environmental control. All system parameters, including water flow, temperature, dilution rate and nutrient concentration, were optimized for obtaining fast, heavy and reproducible biofilm growth. The system make-up water was kept at constant oxygen saturation (by continuous sparging of air), temperature, and pH conditions. Thus, any changes in DO concentrations or pH levels in the recirculating water were considered due to biofilm activity. All monitoring and control parameters were calculated in the data acquisition system, which was controlled by a custom designed LabVIEW computer software program. Data was collected every 15 s, with averages calculated and recorded every 3 to 60 min in an EXCEL spreadsheet for subsequent graphical analysis. The program was designed in such a way that the system was able to function continuously under constant conditions for several weeks. Biocide efficacy testing was conducted through analysis and comparison of the shape and values of the corresponding curves of HTR and DO. Analysis included consideration of curve patterns corresponding to biocide treatment, as well as, biofilm recovery (regrowth). Design, parameters and examples of system operation, testing of biocides as well as details of inocula preparation, growth and test conditions were described earlier (Ludensky, 1998; Ludensky et al., 1998).

3.2. Growth conditions

The sheathed *Sphaerotilus natans* (ATCC 15291), which is known to form a tenacious biofilm on heat exchanger surfaces in cooling water systems and papermaking machines, was selected for biofilm growth. Inocula was pumped into the microbial growth reactor and usually allowed to sit at room temperature overnight. The next day, the make-up water (Clinton, NJ tap) and nutrient (CGY media) were started. Selection of initial growth conditions and parameters of the system was based on previous experience, laboratory limitations, geometric size of the system's components, and the desire to promote a growth of biofilm. Shifting of growth conditions from planktonic growth to attached filamentous growth was obtained by lowering media concentrations to less than 5% and maintaining dilution rates higher than maximum specific rate.

3.3. Treatment program

Monitoring of biocide efficacy was performed on already established biofilm. The treatment procedure was designed in such a manner that the first treatment in each test was performed at the point when HTR slope rose to the level of $2.0–3 \times 10^{-3}$ hr-sq ft-F/BTU. The system was continuously fed with nutrient and makeup water (constant chemistry, oxygen and temperature), and biocide treatment was carried out by an initial slug dose, followed by a continuous, 3-h treatment at a constant concentration based upon the makeup water rate. Such treatment was repeated on sequential 24–72 h intervals. Biocides were prepared as 0.1–0.2% solutions.

Tested biocides were:

- Alkyl dimethyl benzyl ammonium chloride (ADBAC);
- 5-chloro-2-methyl-3-isothiazolone(CMI)/2-methyl-3-isothiazolone (MI) mixture at a 3:1 ratio;
- Mixture of 1-bromo-3-chloro-5.5-dimethylhydantoinhalohydanto, 1,3-dichloro-5,5-dimethylhydantoin, and 1,3-dichloro- 5-ethyl-5-methylhydantoin (Dantobrom RW);
- glutaraldehyde,
- isothiazolone/decyl isononyl dimethyl ammonium chloride (DIDMAC) mixture at 1:3 ratio.

Biocides were dosed in the concentrations common for water treatment applications (as active ingredients): ADBAC: 30 ppm, glutaraldehyde: 100 ppm, isothiazolone mixture (CMI + MI) : 4 ppm, and Dantobrom: 10 ppm. The blend was tested at following concentrations: isothiazolones mixture (4 ppm) + DIDMAC (12 ppm). Disinfectant/cleaner was used in experiment 6 at 2% concentration as a slug dose. Alkaline cleaner was used in Experiment 7 at 2% concentration as a slug dose.

4. Results

4.1. Experimental

A series of experiments was conducted to determine biocidal efficacy of non-oxidizing biocides and their blends on *S. natans* biofilm. Results obtained during these experiments are now described.

Experiment 1 (ADBAC, 30 *ppm).* The established biofilm was treated with ADBAC at 30 ppm using the intermittent regime for two consecutive days. The third treatment was conducted approximately 3 days after the second treatment (Fig. 1).

Experiment 2 (isothiazolones mixture, 4 *ppm).* The established biofilm was treated with isothiazolones at 4 *ppm* using the intermittent regime at 72, 96 and 120 h (Fig. 2).

Experiment 3 (glutaraldehyde, 100 *ppm).* The established biofilm was treated with glutaraldehyde at 100 ppm using the intermittent regime at 96, 120, 144, and 168 h from the beginning of the test (Fig. 3).

Experiment 4 (halohydantoin, 10 *ppm).* The established biofilm was treated with halohydantoin at 10 ppm using the intermittent regime at 121, 145 and 168 hours from the beginning of the test (Fig. 4).

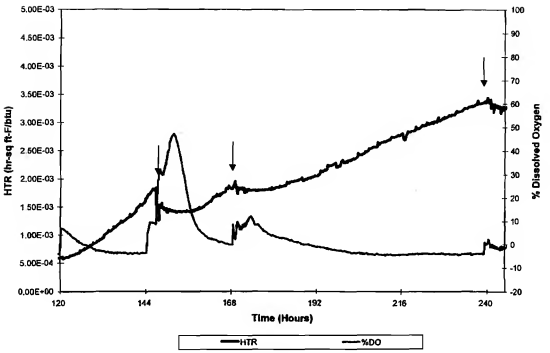

Fig. 1. Testing biocidal efficacy of ADBAC.

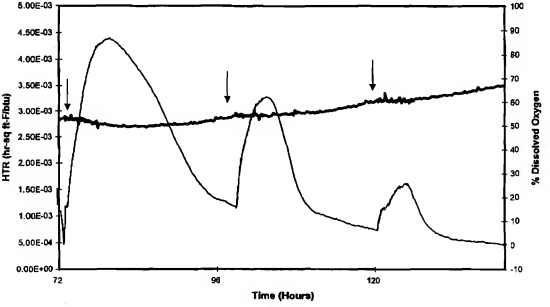

Fig. 2. Testing biocidal efficacy of isothiazolones.

Experiment 5 (*isothiazolones, 4 ppm+DIDMAC, 12 ppm*). The established biofilm was treated with isothiazolones at 4 ppm and DIDMAC at 12 ppm in the intermittent regime at 97, 124, and 167 h from the beginning of the test (Fig. 5).

Experiment 6 (*Disinfectant cleaner, 500 p pm as quat*). The mature established biofilm was treated with Lonza disinfectant cleaner at 2000 ppm using the slug treatment regime at 143 h from the beginning of the test (Fig. 6).

Experiment 7 (*Alkaline cleaner, 2% as product*). The mature established biofilm was treated with Lonza alkaline cleaner (2000 ppm) at pH 11.5 using the slug treatment regime at 293 h from the beginning of the test (Fig. 7).

4.2. Analysis

Experimental curves were analyzed according to the following procedure: three parameters were measured from

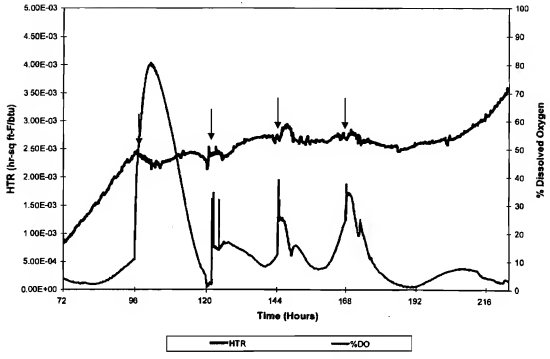

Fig. 3. Testing biocidal efficacy of glutaraldehyde.

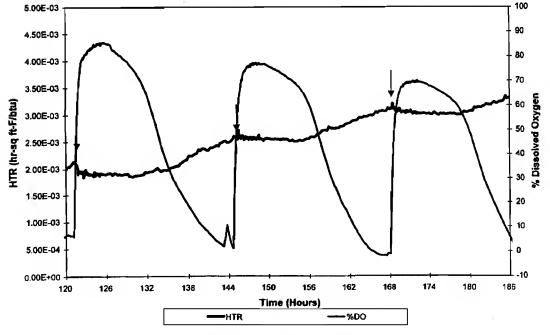

Fig. 4. Testing biocidal efficacy of halohydantoin.

curves corresponding to a biofilm's response to biocide treatment: time of HTR recovery to pretreatment level (RT); maximum (peak) DO level (curve height or H), and the time interval between slopes of DO curve at the 50% of peak DO level (curve width or W). Additionally, the biofilm respiration control coefficient (RC) was calculated as

$RC = 0.5H \times W$, where H is the maximum DO level peak in each treatment; W is time interval between slopes of DO curve at the 50% of peak DO level.

Results of the analyses of the experimental graphs (three consecutive treatments from experiments 1–5) are presented in Table 1. Two main parameters, RT and RC, are

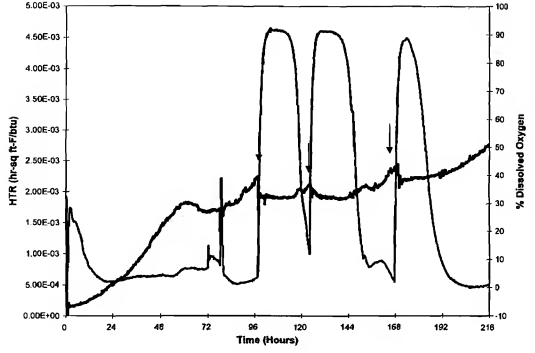

Fig. 5. Testing biocidal efficacy of isothiazolones+DIDMAC.

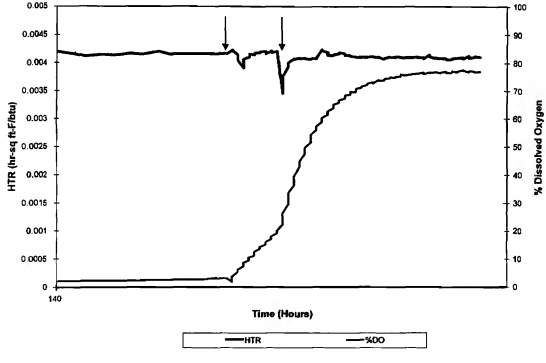

Fig. 6. Testing biofilm removal efficacy of disinfectant cleaner.

of major importance in the interpretation of experimental results. The first parameter, RT, relates to biofilm accumulation (deposit) on the surface (dead or alive), and the second parameter, RC, relates to the amount of oxygen not consumed by biofilm due to a biocide's presence, thus measuring biological activity of a biocide. The height of the DO curve in the RC index corresponds to the level of maximum respiration inhibition, and the width of the DO curve corresponds to the longevity of the biocidal effect. Thus the multiplication of these two factors, H and W,

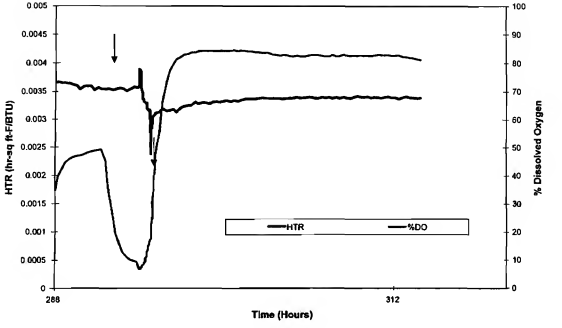

Fig. 7. Testing biofilm removal efficacy of alkaline cleaner.

Table 1
Comparative efficacy of biocides on biofilms

Parameters	Biocide treatments														
	ADBAC			Isothiaz.			Glutarald.			Iso./DIDMAC			Halohydant.		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
RT (h)	20	15	12	24	5	5	15	10	24	27	30	32	15	12	12
W (h)	9	9	3	15	8	5	15	16	8	21	23	16	12	12	14
H (%DO)	46	12	6	86	62	25	80	17	25	92	92	88	86	78	70
RC	207	54	9	645	248	63	600	136	100	966	1058	704	516	468	490

corresponds to the total effect of biocide on biofilm respiration (RC).

All tested biocides showed some level of biocidal efficacy. One common feature for non-oxidizing biocides in their action on the *S. natans* biofilm was the strong HTR and DO response due to the first treatment. Secondly, experiments demonstrated a steady reduction of biofilm response for all three non-oxidizing biocides. Experimental results revealed that biofilms exposed to non-oxidizing biocides show substantially lower response levels to the same biocide on subsequent exposures 24–72 h later. For example, from HTR recovery data (RT), ADBAC controlled biofilm for about 20 h in the first treatment, 15 h in the second treatment, and 12 h in the third treatment. ADBAC respiration control (RC) data (207, 54, 9) demonstrates a similar trend, as well as RT and RC data for isothiazolones and glutaraldehyde. Comparing the efficacy of non-oxidizing and halohydantoin (oxidizing biocide) demonstrates some differences between these two types of biocides. The major feature of oxidizing biocides is the absence of a strong decline in the efficacy during consecutive treatments. Biofilm response results for halogenated hydantoins show only a slight decline in HTR recovery time and respiration rate during three consecutive treatments. Therefore continuous treatment with oxidizing biocides will provide better biofilm control than slug or even slug/continuous dosages of non-oxidizing biocides.

Experimental study shows that the combination of non-oxidizing biocides was more efficacious in biofilm control than biocides when used alone. Simultaneous use of isothiazolones and DIDMAC (which biofilm control efficacy is similar to that of ADBAC) demonstrated better biofilm control than either isothiazolones or ADBAC alone. This biocidal combination controlled biofilm (from HTR data) for about 27, 30, and 32 h after three consecutive treatments. The biofilm DO response (966, 1058, 704) was much more significant than any separately tested biocide and was similar to the test (previously published data) when isothiazolone was used together with oxidizing biocide (Ludyanskiy and Himpler, 1997). Our current laboratory results confirm practical (field) evidence that combinations

of biocides affect biofilms more effectively than biocides used alone.

An important practical aspect of biofilm control with non-oxidizing biocides is their inability (in tested concentrations) to remove biofilms from the surfaces. Being significantly inhibited by non-oxidizing biocides, treated biofilms stayed on the surfaces and demonstrated significant biofilm regrowth, usually within several hours. It is suggested that the control of already established biofilms with non-oxidizing biocides alone is rather impractical. Probably, non-oxidizing biocides are better suited for prevention of biofilm growth or to supplementing oxidizing biocides. Removing capabilities of oxidizing biocides are usually higher when compared to non-oxidizing formulations. Likely, the best approach in biofilm control is to use combinations of non-oxidizing and oxidizing biocides.

Experiments 6 and 7 demonstrated that even high concentrations of disinfectant cleaner and alkaline cleaner were not able to remove biofilms from surfaces. These experiments have significant practical consequences for plant hygiene during manufacturing of household/cosmetics consumer products. Usage of regular disinfectants and cleaners will not provide control of microbiological problems and contamination at the manufacturing plant. Achieving plant hygiene should be done through combination of mechanical cleaning/washing of plant equipment with sanitation procedures.

5. Discussion

Due to the enormous structural and functional complexity of biofilms, a complete explanation of the results obtained in this study is extremely difficult and probably lies beyond the present state-of-the-art understanding of biofilm. We can only hypothesize that there are multiple reasons for recorded biofilm responses to biocides. It is recognized that biocide efficacy depends on several factors including: transportation, adsorption, diffusion, penetration, and interaction at the target site. The differences in biocide efficacy also depend on the mode of action, chemical constitution and chemico-physical properties of biocide as well as on the chemistry of the medium and conditions of biocide application. Depending on the nature of a biocide, any of these parameters could be of major importance. For example, in the case of halogens, it was demonstrated that chemical reaction is the limiting stage of successful transport of biocide to the microbe cell (LeChevallier et al., 1988). Although transport limitation can explain biofilm resistance to free halogen donors, it is thought to be less important for non-oxidizing biocides. It was proposed that altered physiology could be a major mechanism in the case of non-oxidizing biocides, when the outcome of any biocidal treatment directed towards a biofilm will reflect not only the limited access of the treatment agent, but also the susceptibility of the most resistant phenotype. It is generally recognized that increased tolerance of bacterial communities to biocides is the result of phenotypic changes brought about in the surviving population. These changes might involve the induction of multiple antibiotic resistance operons or of other global regulatory systems that respond to sub-inhibitory concentrations of biocides (Brown and Gilbert, 1993). For example, it was shown that bacteria from cooling water systems developed resistance to quats, isothiazolone and thiocarbamates (Brozel and Kloete, 1991). However, in this study resistance was induced by exposing isolates to increasing sublethal concentrations for a period of 10 weeks. Much faster changes in biofilm response were observed in our experiments. The changes in limiting nutrient and growth rate in the deeper layers of the biofilm structure following each biocidal treatment could be important reasons for the observed phenomenon of rapid biofilm recovery. However, several other mechanisms could be considered to assess the reduced sensitivities of bacteria in biofilms to biocides (Nichols, 1989).

6. Conclusions/practical consequences

(1) Non-oxidizing biocides in tested concentrations were not able to remove biofilms from the surfaces. After being significantly inhibited, biofilms recovered rapidly. This class of biocides is not a good choice for biofilm removal/cleaning. It can be used as an additional disinfectant/sanitizer after mechanical/chemical cleaning surfaces from biofilm.

(2) Experiments have revealed that biofilms exposed to non-oxidizing biocides showed a decrease in their response to the same biocide on subsequent exposures 24–72 h later. Therefore, slug dosage of non-oxidizing biocides to control biofilm/slime in cooling waters and papermaking is not the best option for these applications. Continuous application of these biocides is usually rather costly. Therefore, oxidizing biocides might be better choice for continuous or periodic treatments. Another factor to be cautious while using non-oxidizing biocides for biofilm control is a potential resistance to biocides, which may become a major factor, especially in the process of manufacturing of household/cosmetics consumer products.

(3) Combinations of non-oxidizing biocides and oxidizing and non-oxidizing biocides demonstrated better biocidal efficacy against biofilms than when used alone. These combinations might be used successfully in cooling water biofilms, especially dealing with Legionella, as well as in paper making during microbiological upsets, and for especially significant contamination problems in manufacturing of household/cosmetic consumer products.

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